TRACE ELEMENT PROFILE OF B-16 MURINE MELANOMA BY PARTICLE-INDUCED X-RAY EMISSION ANALYSIS

BY

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The primary objective of this research was to characterize the elemental composition of cancerous tissues, and specifically to investigate whether differences exist in the elemental concentrations of primary and metastasized tumor tissue. The analytical technique used was particle-induced x-ray emission analysis.

The first step in achieving this objective was to produce the cancerous tumors in C57BL/6j black mice. B-16 murine melanoma was selected as the tumor model for this research because its biology is well documented in the literature and it has been used extensively in other metastasis research. Primary tumors developed at the site of the subcutaneous injections and metastatic lung tumors grew following the intraveneous injections.

Approximately 30 days following the injections, the mice were sacrificed, and primary and metastatic tumor samples were collected and analyzed. Blood serum was also collected and analyzed to determine

whether changes in serum levels could be correlated with the presence or extent of the disease. Some of the samples were split into two parts prior to analysis so that another research team could perform neutron activation analysis, a complementary analytical technique.

Eight elements (potassium, calcium, iron, copper, zinc, selenium, bromine, and rubidium) were detected in all samples except serum where the rubidium concentration fell below the lower limit of detection. Five of the elements (iron, zinc, selenium, bromine, and rubidium) were observed by the two analytical methods. The results of both techniques corroborated for all elements except zinc.

The Student's t test showed a significantly higher concentration of three elements (calcium, copper, and bromine) in the primary tumor compared to the metastatic tissue.

In humans, calcium and bromine are two of the most abundant elements in the body. Calcium is utilized in the formation of bones and teeth, but bromine has not been found to be essential to proper health and nutrition. Their occurrence in higher proportions in the primary tumor may indicate that the body's defense mechanism uses these elements to combat the tumor.

Copper is an essential element in humans and has been reported at elevated levels in the serum of certain cancer patients. Data from this research confirm the belief that copper is involved in cancer growth, but whether it is used by the body as a therapeutic agent or whether it is required by the rapidly dividing tumor is not known.

This research has shown that elemental concentrations are different in primary and metastasized cancer tissues. Continued research is planned to further investigate differences in elemental content of cancer tissue.

CHAPTER I

INTRODUCTION

Overview

Deaths from cancer have risen dramatically in the United States since the year 1900. Paradoxically, the rise is due, in part, to advances in other areas of medical science and technology, which have greatly enhanced the detection and control of infectious diseases and, thus, increased longevity. Presently, cancer mortality is approximately 400,000 annually and, if the current trend continues, the American Cancer Society predicts in excess of one-half million deaths annually by the year 2000. In the United States, deaths from cancer are surpassed only by those which result from cardiovascular disease and renal disease (Rubin 1978).

More than 270 varieties of tumors have been identified in humans. However, between the two broad manifestations of tumors, solid and liquid, the solid tumors are the leading type which results in death both in the United States and around the world (Carter 1973). Death is not so much the result of the primary tumor, which in many instances may be surgically excised or treated by radiation or chemotherapy, but rather due to metastasis, that insideous ability of malignant cells to detach themselves from the original tumor, spread to distant sites in the body, and form new tumor colonies. Unfortunately, the metastatic colonies are much more difficult to detect and eliminate, so even though

the primary tumor may have been removed, these metastases may continue to proliferate. According to Sugarbaker and Ketcham (1977), approximately 50% of all cancers have metastasized at the time of diagnosis. Nicolson said that " . . . metastases can well considered the conclusive event in the natural history of cancer"(Nicholson 1978b, p. 66). At one time, the focus of cancer treatment emphasized the removal or destruction of the primary tumor; however, now an equal emphasis is being placed on the detection and elmination of metastases.

Clinical Documentation

The clinical documentation of all types of cancers is extensive and statistics on probability of recurrence or 5-year survival rates are precise if the staging is correct. Staging refers to the clinical evaluation of a specific development or distinct phase of the disease, i.e., whether metastasis has occurred or whether regional lumph nodes are involved. However, in spite of all the statistics and knowledge about surgery, radiotherapy, chemotherapy, and immunotherapy, or the many permutations of these treatment choices, the exact cause of cancer and metastasis remains unknown. Clinical experience has proven that metastasis, rather than the primary tumor, is more often the threat in cancer. Therefore, if "cure" rates in cancer patients (with a metastasizing-type tumor) are to be increased, the knowledge and understanding of the biology and those factors which affect metastasis must be increased.

Trace Elements

One possible factor which may affect metastasis, or the area of the body to which cancer spreads, that has not been extensively studied is the role of trace elements. Although trace elements, such as copper, iron, iodine, cobalt, zinc, chromium, selenium, and cadmium, comprise

less than 0.01% by weight of the total-body composition, many are known to be essential components of biological systems (Schwartz 1975). Adverse effects have been observed in humans and animal studies resulting from either a deficiency or excess of certain trace elements. For example, growth retardation has been reported from insufficient zinc intake (Sandstead et al. 1970) and excessive exposure to nickel or beryllium compounds has been correlated to toxic effects or even carcinogenesis (Schwartz 1975). Certain trace elements and their biological roles, if known, are discussed in Chapter II.

MEDLARS and CANCERLIT

A computerized literature search through MEDLARS (Medical Literature Analysis and Retrieval System) and CANCERLIT (Cancer Literature on-line), both of which access the National Library of Medicine data base, showed that the role of trace elements in the metastatic process has not been investigated. Several references were given on studies which identify certain chemical elements as carcinogens or on the use of some elements in the treatment of cancer, but not specifically on investigating differences in trace element concentrations between primary and disseminated tumor.

Primary Tumors And Trace Elements

Evidence of a link between trace elements and primary tumors was established by Guffey et al. (1978) from a study in which trace element concentrations were determined in human tumor tissue and normal tissue within the same organ. They obtained specimens from several different organs, including kidney, lung, colon, breast, larynx, liver, and aorta. By comparing tissues within the same organ, trace element variations within individuals which may be present due to differences in age, sex, background environment, medications, etc. were eliminated and direct comparisons were possible. Their results indicated that some significant

differences exist in elemental concentrations between the tumorous and normal tissues. For example, in a kidney specimen there was no detectable cadmium in the tumor; whereas, it was present in the normal tissue. Also, the amounts of zinc and rubidium were lower in the kidney tumor compared to normal. Another fact reported was that the zinc to copper ratio was lower in kidney tumor versus normal tissue, but similar zinc to copper ratios in breast cancer and normal breast tissue showed the opposite relationship. Differences in trace element concentrations were also observed in the other organs studied.

Metastatic Tumors and Trace Elements

There were only a few references cited in which trace element concentrations were measured in metastatic tumors and these were in humans. Due to the differences in elemental concentrations in humans caused by medications, diet, environment, etc., little more than trends for specific patients can be extracted from these studies (Schwarts 1975). It was reported that metastases in liver, kidney, or lung contained a higher zinc content than normal tissue or the primary tumor (Wright and Dormandy 1972). However, Kew and Mallett (1974) found that hepatic metastases contained lower zinc levels than normal liver.

Epidemiological studies have been conducted which showed correlations between trace metals and cancer. Berg and Burbank (1972) compared concentrations of trace elements in water supplies with cancer mortality and linked nickel with cancer of the mouth and intestine, arsenic with larynx and eye cancers, beryllium with bone, breast, and uterus cancer, and lead with lymphoma, leukemia, and cancer of the gastrointestinal tract and the ovaries. Significantly lower selenium levels were measured

in the serum of cancer patients, especially those with gastrointestinal cancer, compared to serum in healthy individuals (Underwood 1977). Cancerous tissue from the breast and lung were found to have elevated levels of zinc (Santoliquido et al. 1976).

The evidence clearly indicates that an interrelationship exists between trace elements and cancer, but the specifics of the relationship are not known. The phenomenon of cancer metastasis is complex and the establishment of a tumor colony distant from the primary tumor is the end result of several events. Metastasis research is going on in many different directions, which is indicative of the little amount known about its mechanism (See the supplementary bibliography).

Chapter III discusses the experimental set-up of the analytical technique and the induction, collection, and preparation of tumor specimens. C57BL/6 mice were selected for the production of tumors because of the availability of a tumor cell line for which they are the syngenic host. A discussion of the tumor model and its host is in Chapter II.

Objective

The object of this research was to contribute to the overall knowledge of cancer metastasis by providing oncologists with elemental profiles of a biologically well documented tumor model, the B-16 murine melanoma. Primary emphasis was directed toward discerning differences in trace element concentrations between primary tumors and metastases. The analytical measurement technique used was particle-induced x-ray emission analysis. Most samples were split and trace element content was also determined, in a parallel research project, by the complementary method of instrumental neutron activation analysis (INAA) (Swanson 1980). Levels of trace metal concentrations in serum and lungs of both tumor-bearing and control mice were also measured.

The results are contained in Chapter IV. Chapter V discusses some conclusions which may be drawn from the data.

CHAPTER II

BACKGROUND

Trace Elements and Cancer

> The life processes of every living cell are conditioned by its content of trace elements. (Allaway 1965, p. 67)

The fact that "trace elements" have an essential role in the normal functioning of living cells is indisputable. The increasing awareness of the importance of minute quantities of elements in biological systems has stimulated the refinement in the past decade, of chemical analysis at extremely low concentrations. The sensitivity of an analytical technique is a measure of its ability to discern the differences between very small amounts of a substance (Morrison and Skogerboe 1965). At present, sensitivities in the parts per million (ppm) and parts per billion (ppb) range are common.

The term "trace element," as a description of the many mineral constituents which occur in living tissues, arises more from historical usage rather than from a formal definition. Early researchers in the field were unable to measure precisely the concentration with the analytical methods then available, and often described the elements as occurring in "traces" and the name has continued (Underwood 1977).

It is impossible to find a uniform classification for the trace elements or to find a clear distinction between those and the so-called major elements. Morrison and Skogerboe (1965) use the term trace to denote concentrations in the range of 1 to 100 ppm and ultratrace for

concentrations less than 1 ppm. In addition to the accurate quantification of trace elements in biological systems, a more meaningful classification is whether the element is essential to some normal life process. Cotzias (1967) has defined an element as being essential if it meets the following criteria: "(1) it is present in all health tissue of all living things; (2) its concentration from one animal to the next is fairly constant; (3) its withdrawal from the body induces reproducibly the same physiological and structural abnormalities regardless of the species studied; (4) its addition either reverses or prevents these abnormalities; (5) the abnormalities induced by deficiency are always accompanied by pertinent, specific biological changes; and (6) these biochemical changes can be prevented or cured when the deficiency is prevented or cured" (Underwood 1977, p. 2).

Trace Element Needs and Tolerances in Humans

According to Underwood (1977), 26 naturally occurring elements are known to be essential to life. Eleven are classed as major elements, namely carbon, hydrogen, oxygen, nitrogen, sulfur, calcium, phosphorous, potassium, sodium, chlorine, and magnesium. The remaining 15, generally accepted as trace elements are iron, zinc, copper, manganese, nickel, cobalt, molybdenum, selenium, chromium, iodine, fluorine, tin, silicon, vanadium, and arsenic. There are another 20 to 30 elements which have not been found to meet the criteria for essentiality but which appear in varying concentrations in living tissues. Among these are aluminium, antimony, cadmium, mercury, germanium, rubidium, silver, lead, gold, bismuth, titanium, and zirconium (Underwood 1977). Even though they are not essential for the proper functioning of a cell, their presence in elevated levels may have toxic effects. For example, deleterious effects from prolonged exposure to mercury have been observed in goldsmiths

and mirrormakers, and the term "mad as a hatter" derives from symptoms shown by workers in factories treating furs with mercuric nitrate (Underwood 1977). Another element, cadmium, although tolerated by the body in relatively low concentrations, is toxic to almost every system in the animal body, whether ingested, injected, or inhaled (Underwood 1977).

A property common to all trace elements is that they normally occur in living tissues at low concentrations. These normal concentrations are characteristic for each element within the tissue type. For example, the "normal" level of zinc in brain tissue of man is 14 ppm, whereas the level in the prostate is typically 102 ppm. The usual whole-body copper concentration in human adults is 1.7 ppm. The concentration range for each essential element is rather narrow and must be maintained within these limits if the functional integrity of the tissues is to be preserved. Prolonged exposure to an environment, or ingestion of a diet, either deficient or excessively high in a particular element will result in imbalances within the body and cause the body burden to fall outside the "normal" functional range. In these cases, biochemical changes occur, physiological functions are altered, and "structural disorders may arise in ways which differ with different elements, with the degree and duration of the dietary deficiency or toxicity, and with the age, sex, and species of the animal involved " (Underwood 1977, p. 7).

The Role of Trace Elements in Humans

Although the reasons are still unknown, it is evident that certain elements have been selected for the vital processes of living cells. It is interesting to note that of the 26 elements now known to be essential

for life, only three, i.e., iodine, tin, and molybdenum, have an atomic number (Z) greater than 34, and the greatest proportion lie in the range between 23 and 34 (Underwood 1977, ICRP No. 23 1975). However, within this range are found gallium and germanium, for which no essential roles are known.

The principle function of trace elements in living cells is to act as a catalyst. In doing so, their roles range from providing weak ionic effects to highly specific interactions as metalloenzymes (Underwood 1977). It may be through their role as a catalyst that trace elements also contribute to or influence cancer metastasis.

Selected Trace Elements

The trace elements discussed below were chosen because they are the most common biological samples and are easily measured by the analytical technique utilized in this research.

Potassium. In combination with other minerals in the body, potassium forms alkaline salts which are essential in maintaining the acid-base and water balance in the body. All body cells, especially muscle tissue, require a high potassium content. A proper balance between potassium, sodium, and calcium in the blood plasma is necessary for proper cardiac function. Potassium is found in abundance in all biological samples, primarily as an intracellular cation (Miller and Keane 1978).

Calcium. Calcium is the most abundant mineral in the body. In combination with phosphorus it forms calcium phosphate, the dense, hard material of the bones and teeth.

A small, constant level of calcium is necessary in the blood for some important body functions such as maintenance of the heartbeat, clotting of the blood, and the normal functioning of muscle and nerves.

Iron. A normal, adult man is estimated to contain 4 to 5 g of iron or 60 to 70 ppm of the whole body of a 70 kg individual. However, it has been found that the iron concentration in animals varies considerably with the species, age, sex, diet, and state of health (Underwood 1977). Generally, iron is found in the body in a bound or complex state rather than ionic, such as bound to protein in hemaglobin. Hahn (1937) estimated blood hemoglobin iron to be 60 to 70% of the total body iron in humans and free ionic iron to be present in negligible quantities.

The distribution of iron within the body organs is quite variable with the liver and spleen carrying the highest concentrations, followed by the kidneys, heart, skeletal muscles, and brain, in which levels of one-half to one-tenth that of the liver and spleen are found (Underwood 1977). The liver has a remarkably high storage capacity for iron. In some disease states, such as malignancy or chronic infection, up to 10 g of iron may occur in the liver (Hussain and Patwardhan 1959).

Copper. The concentration of copper varies in humans depending upon age. Normally, newborns have higher levels of copper per unit of body weight than adults, 4.7 ppm versus 1.7 ppm (Underwood 1977). The healthy adult body has been estimated to contain a total of 80 mg of copper (Cartwright and Wintrobe 1964). Copper is necessary for bone formation and for the formation of blood because it acts as a catalyst in the transformation of inorganic iron into hemoglobin (Miller and Keane 1978). A diet deficient in copper can lead to anemia, probably due to copper's role in hemoglobin production (Allaway 1965).

Elevated serum copper levels have been reported in a variety of diseases, both cancer and noncancer. In 236 patients having malignant lymphomas (excluding Hodgkin's disease), Hrgovcic et al. (1973a) measured significant differences in serum copper levels which were related to the

activity of the disease. Levels were high in patients before treatment, but following successful therapy, the concentrations decreased to levels found in "normal" patients. Similar trends have been observed in adult patients with Hodgkin's disease (Hrgovcic et al. 1973b), and other forms of cancer including bronchogenic carcinoma (Tani and Kokkola 1972), squamous cell carcinoma of the larynx, cervical and other gynecological cancer, bladder and breast cancer, but not prostate cancer (Schwartz 1975). The addition of cupric acetate to the diet of rats has been shown to inhibit hepatoma induction; however, the mechanism is not understood (Underwood 1977).

Zinc. Zinc has been found to be an essential element in humans and is especially important in infant nutrition (Sandstead et al. 1970). Its role in the functioning of certain metalloenzymes has been extensively studied.

Low zinc levels have been observed in the serum of patients with cancer of the bronchus and colon, but not in other forms of cancer (Davies et al. 1968, Schwartz 1975). The data on zinc concentrations in tissues of cancer patients have been variable. One study showed that metastases in the liver, kidney, or lungs contained more zinc than the corresponding normal tissue or the tumor itself (Kaltenbach and Egen 1968, Wright and Dormandy 1972, Schwartz 1975). However, low zinc concentrations have been reported in hepatic metastases from several sources with greater than normal values in the liver harboring the primary tumor (Schwartz 1975).

A correlation between excessive zinc ingestion and cancer of the esophagus and stomach has been reported (Stocks and Davies 1964, McGlashan 1972). Yet, Poswillo and Cohen (1971) found that ingestion of zinc inhibited the development of tumors.

One very well documented observation is the influence of zinc on wound healing. In patients with zinc deficiency, there is a demonstrable delay in healing (Hrgovcic et al. 1968), whereas, healing was significantly accelerated in postoperative patients who were administered oral doses of zinc (Poires and Strain 1966, Poires et al. 1967a, Poires et al. 1967b, Hallbook and Lanner 1972).

Selenium. Selenium is an essential element which occurs in all cells and tissues of the animal body in variable concentrations, depending upon the chemical form and dietary intake. Generally, the liver and kidneys contain the highest concentrations with much lower levels in muscles, bones, and the blood (Underwood 1977).

In an early study by Nelson et al. (1943), selenium was implicated in the production of liver cancer in rats, but more recent investigations have shown that selenium can produce toxic hepatitis which resembles cancer (Harr et al. 1967). It is now established that dietary selenium is capable of preventing tumors in animals fed or subjected to topically administered carcinogens (Schwartz 1975). The specific role of selenium in inhibiting tumorigenesis is not known. It is known to bind loosely to proteins and that it is taken up rapidly by fast-growing tumors. It may be that selenium, in a tumor cell, competes with the carcinogen for binding sites of the protein and thereby prevents cancerigenesis (Schwartz 1975). The protective role of selenium in cancer formation is supported by a study in which negative correlations were found between selenium levels in blood and mortality from cancer in 10 cities with populations of 40,000 to 70,000 (Shamberger and Frost 1969).

Bromine. Bromine is one of the most abundant trace elements in the biosphere, even though it has not been shown to perform any essential

functions in animals or plants. Its concentration in body fluids and tissues can vary substantially according to the bromine dietary intake (Winnek and Smith 1937, Lynn et al. 1963, Underwood 1977). Cole and Patrick (1958) showed that most animal tissues contain 50 to 100 times more bromine than iodine and that it does not tend to accumulate in any particular organ.

Rubidium. No essential role in the proper functioning of human cells has been found for rubidium, yet it is interesting to note that it appears in higher concentrations in living tissues than can be accounted for by environmental levels. Rubidium very closely resembles potassium, chemically, and it has been proposed that it can act as a nutritional substitute for potassium, at least in lower animals (Underwood 1977). All soft tissues in humans contain levels of rubidium which are high when compared to the other trace elements. The total-body content of rubidium is approximately 360 mg (Yamagata 1962).

Cancer

Superficially, cancer can be described very simply. The common explanation is that some body cell undergoes a change which makes it much less susceptible to the growth-control mechanisms than its surrounding normal cells. It begins to multiply, recreating itself with still more mutant cells. The more rapidly the cancer grows, the more they tend to lose their ability to differentiate, or to specialize, or to adapt to the function they were originally intended. Suss et al. (1973) said that cancer cells devote all their energies to growing instead of functioning. These cells also have the ability to survive in spite of the host's defense mechanisms. This means that once cancer cells have initiated, unless destroyed or removed, they will

continue to multiply and possibly disseminate to other areas of the body, or to metastasize.

Benign versus Malignant

There are two broad categories which describe the nature of cancerous neoplasms—benign and malignant. A benign tumor is characterized as one which remains relatively similar to the surrounding tissue from which it derived. Benign tumors grow slowly by expansion, but remain encapsulated by a layer of connective tissue (Nicolson 1978b). By contrast, a malignant tumor normally grows rapidly, is atypical of the surrounding tissue and does not remain encapsulated. It is a malignant tumor's characteristic ability to invade surrounding normal tissue, and colonize distant sites, via the blood and lymphatic systems, that has resulted in so much research on the biology of metastasis.

The sequential steps in metastasis have been categorized as: (1) extension of the primary tumor into the surrounding tissues; (2) penetration into the blood stream, lymphatic system, or body cavities; (3) release of tumor cells to be transported to distant sites; (4) arrest and reinvasion of normal tissues; and (5) manipulation of the environment to allow tumor-cell survival, vascularization, and metastatic growth (Nicolson 1978b).

Data indicate that although a large number of cells are continually being released from a primary tumor, only a small fraction actually survive the hostile environment during transport to eventually produce metastases (Butler and Guillino 1975, Fidler 1973a).

Theories of Metastatic Distribution

Two long-standing theories have been proposed to explain the distinctive patterns of distribution of metastases. In 1889, Paget (1889)

postulated the "seed and soil" hypothesis which states that the micro-environment of one organ may favor the arrest and subsequent growth of circulating tumor cells while other organs are not suitable. That is, some organs seem to be more "fertile" for the establishment of metastasis. In contrast to that theory, Ewing (1928) advanced the argument that metastasis is influenced purely by "mechanical factors" such as anatomic and hemodynamic factors in the vasculature. Both theories have been shown to have merit, but, for years they were considered as mutually exclusive events. Weiss (1977) states that the theories should not be considered in that manner.

More recently, a third hypothesis has been proposed that credits properties of the tumor cells themselves to influence the pattern of spread and growth of metastasis. A remarkable experiment demonstrated this theory. Kinsey (1960) used a metastasizing murine melanoma which preferentially spread to lung tissue. The tumor was induced subcutaneously. The mice also had lung tissue implanted subcutaneously in a rear leg. When the mice were sacrificed, metastases were found in both the normal lung tissue and that which was implanted in the leg. For controls, fragments from other organs were also implanted in the mice, but, metastases did not appear in the other organ implants. This meant that the circulating tumor cells must "home-in" on lung tissue.

There are, of course, other theories postulated; however, it is not the purpose of this research to delve into the theories.

Tumor Model

One of the goals of this research project was to investigate whether differences exist in the concentrations of trace elements between primary cancer tumor, metastatic tumor, and non-diseased tissue. Another goal was to make the findings relevent to the knowledge of human cancer. A

prime requisite for studying metastasis is to have a predictable model; that is, one for which the biological response (growth of a primary tumor and metastases) is well known and predictable. An equally important consideration, coupled to the biology, is the need for trace element concentrations to be consistent among the populations sampled. Initially, consideration was given to analyzing human tissues received through surgery or autopsy, but studies have shown that there are no "normals" among human populations so far as trace element concentrations are concerned. Factors such as age, sex, diet, environment, and occupation have profound effects on the body burden of trace elements. To eliminate as many of these types of variables as possible, the natural choice was to sample an animal population in which these variables could be selected and controlled. The tumor model selected for this study was the B-16 murine melanoma.

Melanoma

The word "melanoma" comes from the Greek word element, melano, which means black. Melanin is a dark, sulfur-containing pigment normally found in the hair, skin, ciliary body, choroid of the eye, pigment layer of the retina, and certain nerve cells. It occurs abnormally in tumors known as melanomas. A malignant melanoma usually develops from a nevus or mole. It consists of melanin-pigmented cells and has a marked tendency to metastasize (Miller and Keane 1978). Metastatic melanoma is one of the cancers least susceptible to treatment in humans, primarily because of the frequency of lymphatic and blood-borne metastasis. B-16 murine melanoma has the same characteristic.

B-16 Murine Melanoma

The B-16 murine melanoma arose spontaneously in a C57BL/6 mouse in 1954. It is transplantable from mouse to mouse or can be grown in tissue culture. It has been carried forward and extensively studied since that time. It is an excellent model for this investigation because it closely resembles the human malignant melanoma in regard to its non-random distribution of metastasis and its immune reponses to tumor-associated antigens (Bystryn et al. 1974). When B-16 melanoma cells are injected subcutaneously into mice, the melanoma metastasizes at a low to moderate rate. However, in order to achieve a more precise assay of metastatic potential, known quantities of tumor cells are injected intravenously into a lateral tail vein. The cells circulate, arrest, invade, survive, and multiply to form pigmented tumor colonies in the lungs and fewer in the ovaries, body cavities, liver, and brain (Nicolson 1978a).

Experiments Involving B-16 Murine Melanoma

It is its biologically-reproducible characteristic to metastasize that has proved to be the value of B-16 melanoma as a research vehicle. The non-random distribution of metastatic colonies, mentioned above, has been extensively studied and documented. A considerable wealth of knowledge regarding the metastatic processes has been derived from it.

In 1972, Dr. I.J. Fidler conducted a number of experiments relating the number, size, homogeniety, and viability of B-16 melanoma cells to the incidence of metastases. B-16 cells grown in vitro were used. Groups of mice were injected in a lateral tail vein with varying numbers of B-16 cells, some of which had been gamma-irradiated to destroy viability. He found that the number of lung metastases was proportional to the number

of viable tumor cells injected and that mice injected with viable and dead tumor cells had significantly more pulmonary tumors compared with mice receiving viable B-16 cells only. In another experiment, Dr. Fidler injected mice intravenously with single B-16 cells and another group with clumps of four or five cells together. Although the total number of cells injected into each group was constant, a significantly higher number of lung metastases occurred in the group receiving clumps of cells (Fidler 1973a). It must be pointed out that while the lungs are the first capillary bed encountered by tumor cells injected intravenously, the B-16 melanoma inherently metastasizes to lung tissue.

One feature of B-16 melanoma that has made it very popular as a tumor model is that the results can be quantitated. Most other studies on metastasis in laboratory animals were carried out with transplantable tumor cells propagated in vivo. Naturally when harvesting cells, some of the normal cells were selected also, and the viability of the cells varied greatly from onepreparation to another. Since the B-16 melanoma can be grown in vitro, tumor cell suspensions can be standarized for viability and thus metastatic ability. Bystryn et al. (1974) quantitated the latent time to tumor appearance, tumor size, rate of growth of tumors, and survival rate of mice injected subcutaneously in the abdomen.

The question of whether a "mechanical" or "soil" factor in the host determines the site of arrest of malignant cells or whether the cell possesses some characteristic which causes it to seek a particular organ was answered with the B-16 melanoma. A group of mice were injected intravenously with cells from the B-16 melanoma tumor lines, sacrificed 14 days later, and the lung metastases collected. These tumor cells were adapted to grow in tissue culture. When they reached confluency, they were

harvested, prepared into an inoculum and injected into another group of mice. The same cycle was completed again by collecting the pulmonary metastases, reculturing and reinjecting. This cycle was repeated four times. Each time, the number of cells injected was constant, as was the viability. However, the number of lung metastases increased with each cycle, demonstrating that the metastatic process is dependent upon the tumor cell rather than the host.

In order to investigate factors which may affect metastasis, particularly the rates of metastasis, it is necessary to have a biologically reproducible model and at least two tumor cell lines; one which metastates faster than the other. Dr. I. J. Fidler perfected such a model from the B-16 melanoma. In an experiment, identical to the one described previously, Dr. Fidler continued the cycle of injecting, collecting lung metastases, culturing the metastatic cells in vitro, reinjecting, etc. With the completion of the first cycle, the cultured cell line was identified as B-16(F1); after the second passage, B-16(F2), and so on. He continued this for ten cycles and ended up with a highly metastasizing subline labelled B-16(F10). As in the experiment above, the number of pulmonary tumor colonies increased with each cycle. This cell line has been preserved and is available for metastatic research (Fidler 1973b, Fidler 1975a).

Another significant experiment demonstrated that the population of cells in the primary tumor is highly heterogeneous, at least in the B-16 melanoma line. For this experiment, an unselected population of B-16 cells was divided into two parts. The first part was injected intravenously into a group of mice. From the second part, individual cells were isolated at random, cloned, and then the separate cloned cells

were injected intravenously into another group of mice. When the mice were sacrificed, the number of lung metastases were counted. In the first group, very nearly the same number of metastases occurred in each mouse. But in the second group, the number of lung metastases varied widely among the mice. This indicated that not all cells have the same metastatic ability and that a metastasizing-type primary tumor contains a heterogeneous population of cells (Fidler 1975a, Fidler 1975b).

B-16 Melanoma for this Project

The two sublines B-16(F1) and B-16(F10) were furnished by Dr. I. J. Fidler for this investigation. A considerable amount of time was spent in perfecting the tumor cells to grow in tissue culture, preparing the cells into an inoculum, and injecting into the animals. Concurrent with refining the tumor cell and animal portions of the experiment, the analytical technique was being perfected.

Analytical Technique

Introduction

The score of years beginning in 1895 was one of the most epochal in the long history of physics. In that year, Wilhelm C. Roentgen, a professor of physics at the University of Wurzburg, Germany, was attempting to characterize the nature of the recently discovered cathode rays. He was particularly interested in the fluorescence which was produced when the rays impinged on the glass walls of the cathode ray tube. For one experiment, Professor Roentgen covered the entire apparatus with black paper. In the darkened room, with the tube energized, he noticed that a nearby screen of barium platinocyanide was glowing brightly and he rightly concluded that some kind of penetrating

rays were escaping the cathode ray tube, interacting with and causing the illumination of the screen. He reported the discovery of a new kind of rays and named them x rays. In less than a month, x rays were being studied in laboratories around the world.

Usually such discoveries in physics take years to find practical applications, but x rays were used in many medical and industrial situations within the first year.

The beginning of applications of x rays for spectrochemical analyses was found in the discovery by Chadwick (1912, 1913) that x rays were emitted from substances when irradiated by radioactive source. In 1912 came the discoveries that x rays could be diffracted and reflected, which led W. H. Bragg and W. L. Bragg to build the first x-ray spectrometer.

These were the essential, rudimentary discoveries which have led to the present techniques of trace element analysis by x-ray spectrometry.

X-ray emission spectrometry has been used for many years to measure or characterize the elemental profile of various types of samples (Johansson and Johansson 1976). In the past, photons and electrons were used primarily as the excitation sources and the characteristic x rays emitted by this excitation process were analyzed by detector-analyzer systems having much lower resolution than is possible today (Johansson et al. 1972). Then in the mid 1960's, comparisons were made of the production of characteristic x rays by protons, electrons, and primary x rays and it was demonstrated that significantly more x rays could be produced by bombarding targets with charged particles than by protons. The first article on "particle-induced x-rays emission"(PIXE) analysis was published in 1970 (Johansson et al. 1970). In 1970, it was experimentally shown by Watson et al. (1971)

and Flocchini et al. (1972) that the combination of x-ray excitation by protons and detection by a silicon detector constitutes a powerful method for multielemental trace analysis of high sensitivity.

The decade of the 1970's brought new solid state technology which led to radiation detectors and analyzers with significantly improved resolution. Since then, many papers have been published in which the method is discussed on various levels and in which a great number of applications are described. The supplimentary bibliography contains a categorized list of these references.

The purpose of the following section is to review PIXE and its use for analytical purposes. Some practical applications are briefly described to illustrate the usefulness of the technique, followed by a discussion of the physical background, and the basic principles of the method.

Particle-Induced X-Ray Emission Spectroscopy

Immediately following the first reported use of PIXE analysis for the determination of elemental concentrations, several researchers began to develop applications for environmental and biological sampling (Johansson et al. 1970). Studies of airborne particulates have been conducted in several locations by analysis of air filters. A group at Florida State University collected aerosol samples from both coastal and inland locations and was able to determine the distribution and sources of the particulates (Johansson and Johansson 1976). In a similar study in St. Louis, Winchester et al. (1974) were able to correlate changes in elemental airborne concentrations with meteorological changes and the movement of industrial air pollution. Studies have also been carried out in work areas where inhaled aerosols can lodge in the respiratory tract of workers such as in welding operations (Johansson and Johansson 1976). Researchers at Purdue University assessed the use of the PIXE

technique for the multielemental analysis of drinking water samples and concluded that it is a practical method for large scale projects involving health effects, environmental control, mineral resource evaluation, and energy utilization (Simms and Rickey 1978).

Many researchers have used PIXE analysis in medical and biological investigations. Because ofen, very small quantities of material are available to analyze, for example from biopsy, the PIXE technique is a practical method since only 5 to 10 mg of sample are necessary. Lear et al. (1976) analyzed specimens from 15 different organs taken during autopsy of humans who died from a variety of diseases. Their analyses showed that elemental concentrations changed with the age of the population sampled. For example, the calcium level in the kidneys increased with age. Cadmium concentrations in kidney tissues were very low in infants, then increased with age until about 60 to 70 years then began to decline. Human and animal body fluids such as whole blood and serum can readily be analyzed by PIXE. The supplementary bibliography lists several references on medical and biological uses of PIXE analysis.

Applications of PIXE analysis in oncological investigations have primarily centered on comparisons of trace element concentrations between tumors and normal surrounding tissue within the same organ. Kubo et al. (1976) measured trace elements in human liver and reported decreases in the quantity of iron, copper, and zinc in tumor compared to normal liver tissue. Valkovic (1977) found differences in trace element concentrations between the spleen of normal C3H mice and the spleen of C3H mice with radiation-induced fibrosarcoma. The research of Guffey et al. (1978), who compared tumorous with normal tissue in seven different organs, was mentioned in Chapter I. A recent study by Maenhaut et al. (1980) measured

the concentration of trace elements in kidney tumor and found dramatic differences between diseased and normal tissue when comparing ratios of the elements to zinc.

Other applications of PIXE analysis include geological studies of rocks and semi-precious stones (Clark et al. 1975, Van Grieken et al. 1975). PIXE has also been used in forensic sciences (Barnes et al. 1973), archeological investigations (Gordon and Kraner 1972), and for the determination of bromine and zinc levels in wheat flour (Martin et al. 1975).

Basic Principles of PIXE

The principle features of PIXE analysis can best be described by reference to a schematic diagram (Figure 1).

An accelerator is used to produce a beam of charged particles (protons, alphas, or heavy ions) which is directed along an experimental drift tube and made to impinge on the sample target. The system is maintained under a vacuum of approximately 5×10^{-5} to 1×10^{-6} Torr during irradiation by means of a turbomolecular pump and a liquid nitrogen cold trap. In order to homogenize the areal density of the particles on the target, a diffuser foil (usually gold or carbon) is inserted into the beam. Beyond the diffuser foil, a series of collimators is utilized to limit and define the cross sectional area of the beam striking the target. The target is typically a thin foil of carbon or plastic upon which the sample to be analyzed has been fixed. Thick targets, such as sections of organic tissue or powder compressed into a pellet, may also be used (Johansson and Johansson 1976). The particles which do not interact with the target material pass through and are collected in a Faraday cup connected to a beam current integrator.

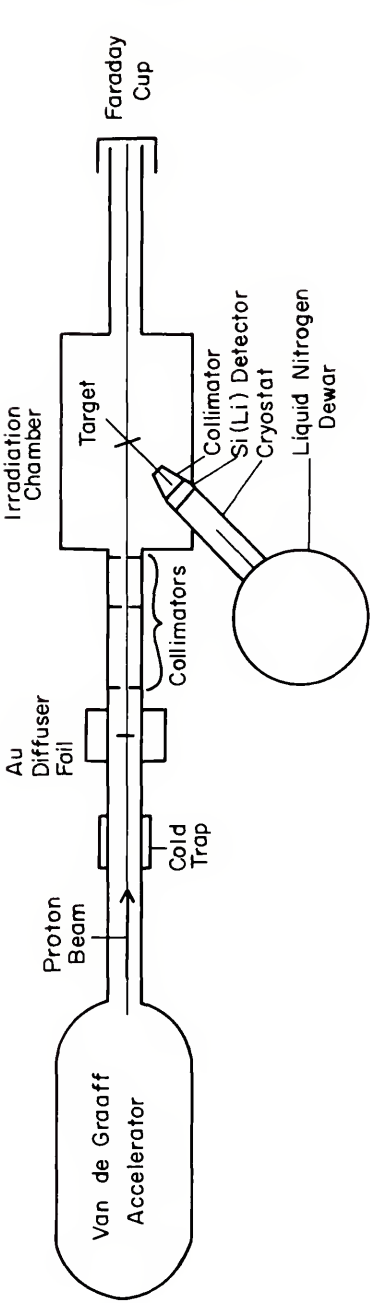


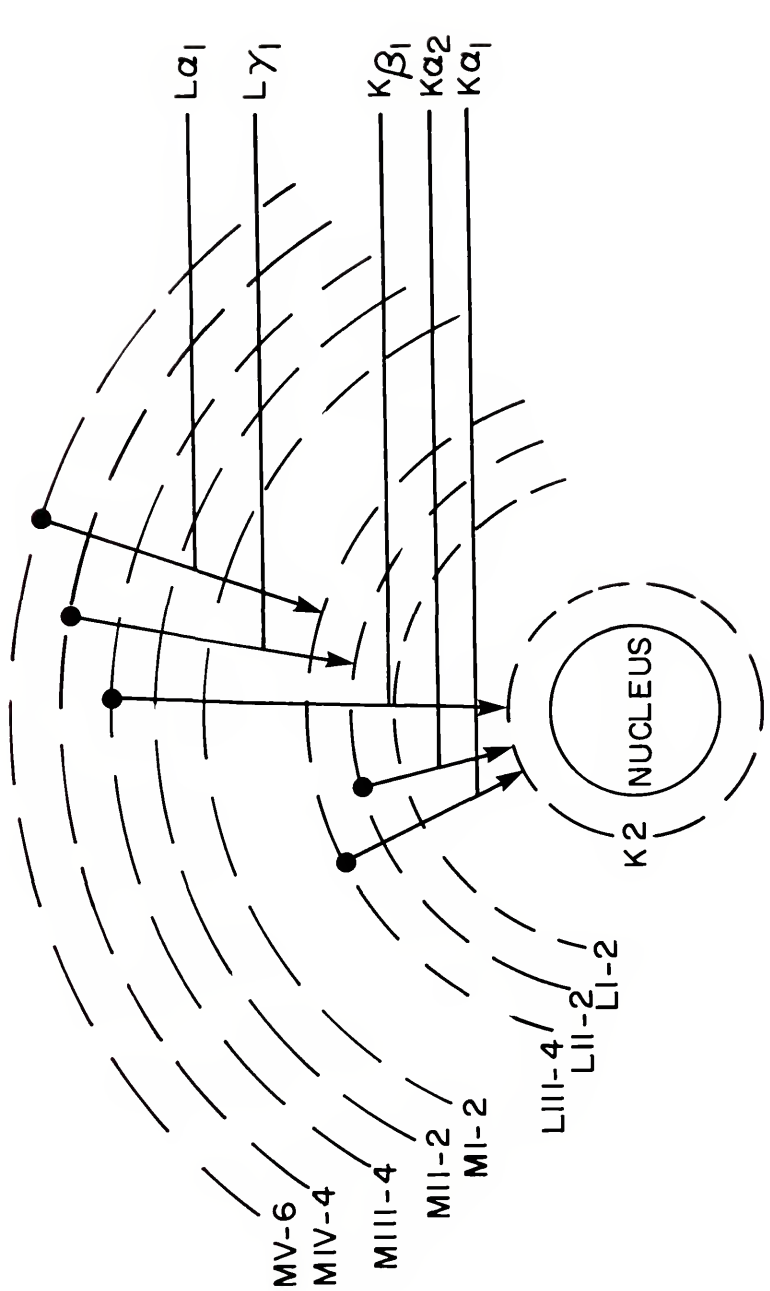
FIGURE 1. SCHEMATIC DIAGRAM OF THE EXPERIMENTAL ARRANGEMENT FOR PIXE ANALYSIS

Those particles which do interact with the sample ionize the target atoms and create electron vacancies in the inner shells (K or L) of the atoms. The atom is then in an energy-rich or "excited" state. In order to return to the ground state electrons within the ionized atom fall or cascade from outer orbitals towards the nucleus to fill the vacancies. Each transition constitutes an energy loss which appears as an x-ray photon. For example, when a K-shell electron is ejected, a series of spontaneous transitions follows; each fills a vancancy in a lower level with resultant emission of an x-ray photon, but also creates a vacancy in a level farther out (Bertin 1978). The continuous bombardment of a sample by ionizing particles results in the simultaneous emission of K, L, and M series x rays for all ionized atoms. Due to the nature of atomic structure, the energies of the x-ray photons are "characteristic" of the specific atom or element, e.g., no two elements have exactly the same characteristic x rays.

For each shell, the characteristic x rays are normally split into groups, e.g. the transitions to the K-shell into a Kα series and a Kβ series depending upon from which orbital the electron originates. The names of the characteristic x rays are designated according to the electron transitions from which they were created. Transitions to the L-shell split into a much larger group of characteristic x rays (Bertin 1978). See Figure 2 for more detail of the nomenclature of characteristic x-radiation. Typical energies of characteristic x rays of interest in biological samples range from approximately 3 to 35 KeV. This includes the K-characteristic x rays for elements potassium (Z=9) through cerium (Z=58). One transition not depicted in Figure 2 which occurs frequently is from the N shell to the K shell. This produces the Kβ2 characteristic

FIGURE 2. SCHEMATIC OF X-RAY SPECTRA NOMENCLATURE.

Examples of intra-atom electron transitions responsible for principle x-ray spectra lines of analytical interest. The number following each orbital symbol (K, LI, LII, etc.) is the number of electrons in that orbital when full.


Lα1
Lγ1
Kβ1
Kα2
Kα1
NUCLEUS
K 2
LI-2
LII-2
LIII-4
MI-2
MII-2
MIII-4
MIV-4
MV-6

x ray, which appears in spectra which have been doped with silver, See Appendix I for some examples.

Detector-Analyzer System

Characteristic x rays are measured by a solid-state, semiconductor, energy-dispersive detector which converts each absorbed x-ray photon into a pulse of electric current. The amplitude of the pulse is proportional to the photon energy. The output pulses from the detector are then amplified and sent to a multichannel pulse-height analyzer where they are electronically sorted into "channels" according to their amplitude. Since the number (or intensity) of x rays of a particular energy emitted from a sample is proportional to the concentration of that element within the sample, the resulting pulse-height distribution is displayed on a cathode-ray tube as peaks on a scale of intensity versus pulse height or photon energy. Qualitative analysis is accomplished simply by observing the peaks present in the spectrum, determining the photon energy corresponding to that peak, via calibration of standards, and correlating the energy to characteristic radiation of a specific element. Concentrations of elements in a target may be caluclated from the integrated intensities of the specific peak in a pulse-height spectrum. Spectrum analysis will be discussed in Chapter III.

The energy of the bombarding particles is typically in the range of 1 to 5 MeV per atomic mass unit and beam currents for biological samples usually range from 10 to 200 nA.

CHAPTER III

METHODS AND MATERIALS

Design of the Experiment

In the original plan of the experiment, all samples collected were to be analyzed by both the PIXE and INAA techniques. Primary subcutaneous tumor was to be compared to metastatic tissue, and serum was to be compared among the mice having subcutaneous tumors, those having pulmonary metastases, and the control mice. The point of analyzing serum for trace elements was to attempt to correlate elemental concentrations with the state of the disease; that is, to test whether serum is a reliable "marker" which could be used to measure the extent of disease. Preliminary studies revealed that the quantity of whole blood which could be obtained from a mouse (usually less than 2 ml) was insufficient for analysis by both techniques. Therefore, it was decided that serum would be analyzed by the PIXE technique only, but that the major emphasis was still toward providing elemental profiles of the primary and secondary cancer tissues.

The mice were divided into three groups with a further split between experimental and control mice within each group. The first group of mice received both a subcutaneous injection and two weeks later, an intravenous injection. By analyzing both tumor types (primary and metastatic) from the same mouse, any variations between mice would be eliminated. However, when the mice were sacrificed a month later, they all had very large subcutaneous tumors but none showed any evidence of

metastases. It was then decided that Group II mice would be injected subcutaneously only and Group III mice would receive only an intravenous injection. The experimental scheme is depicted in Figure 3.

B-16 Murine Melanoma Tumor Cells

All handling of the cells and preparation of culture media was done in a sterile hood, especially designed for tissue culture work.

The work with the tumor cells can be categorized into four general areas: (1) splitting and reculturing; (2) cryopreserving; (3) thawing to reculture; and (4) preparing the cells for injecting into the mice. The specific steps involved in each procedure are listed in Appendices C, D, E, and F, respectively. The original cell lines used in this study were furnished by Dr. I. J. Fidler from the National Cancer Institute, Frederick, MD. There was one vial each of the B-16(F1) and B-16(F10) cell lines, described in Chapter II. The cells arrived unfrozen in culture media. New complete minimum essential media (CMEM - See Appendix B) was prepared and the cells were resuspended and transferred into plastic tissue culture flasks. The cells were incubated at $37^{o}C$ for 2 days before the medium was exchanged.

Splitting and Reculturing B-16 Melanoma Cells

Initially, a problem arose in keeping the cells alive. It was discovered that the extent of confluency to which the cells were permitted to grow was extremely important. The cells grew in a monolayer on the bottom of the flask and if allowed to grow beyond approximately 90% confluency, they began to die rapidly. Figure 4 is a 40X power photomicrograph of B-16(F10) cells at two different phase settings under a phase-contrast microscope (A and B). Photograph C shows the same tumor line under an inverted, regular-light microscope. These photomicrographs

FIGURE 3. EXPERIMENTAL SCHEME

Three groups of C57/BL6j mice were split into experimental and control subgroups. Controls received injections of Hanks' basic salt solution (HBSS) only, while experimental animals received HBSS cellular suspensions of B-16 melanoma. Injection volume was 0.2 ml/mouse. Group I experimental mice received 2.72×10^4 viable B-16(F1) cells/injection subcutaneously. Two weeks later these same Group I mice received 5.00×10^4 viable B-16(F1) cells/injection intravenously in a lateral tail vein. Group I animals were sacrificed 20 to 30 days post subcutaneous injection. Subcutaneous primary tumors were excised from experimental animals along with lungs from experimental and control mice. Group II mice received 6.02×10^4 viable B-16(F1) cells/injection subcutaneously and were sacrificed 23 to 33 days post injection. Primary tumors and lungs were collected. Lungs from mice bearing subcutaneous tumors showed no visible evidence of metastatic growth. Group III mice received 6.10×10^4 viable B16(F10) cells/injection intravenously in a lateral tail vein. Lungs with visible metastatic growth and control lungs were removed 31 to 33 days post injection.

Except for several primary tumor samples, all Group I samples were sent to Gent, Belgium for comparative analysis. Primary tumors from Group II mice were compared in elemental composition with individual lung metastases excised from Group III mice. Serum was also collected from mice in Groups II and III.

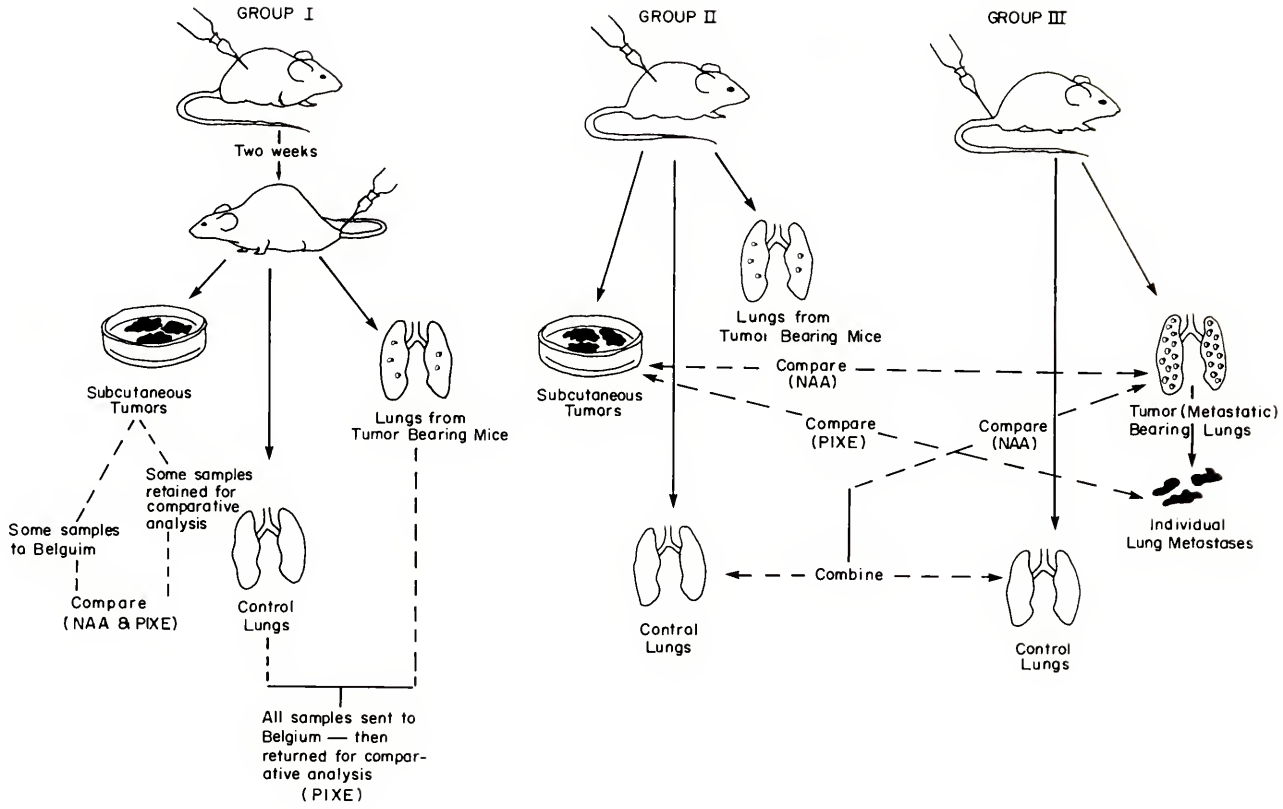
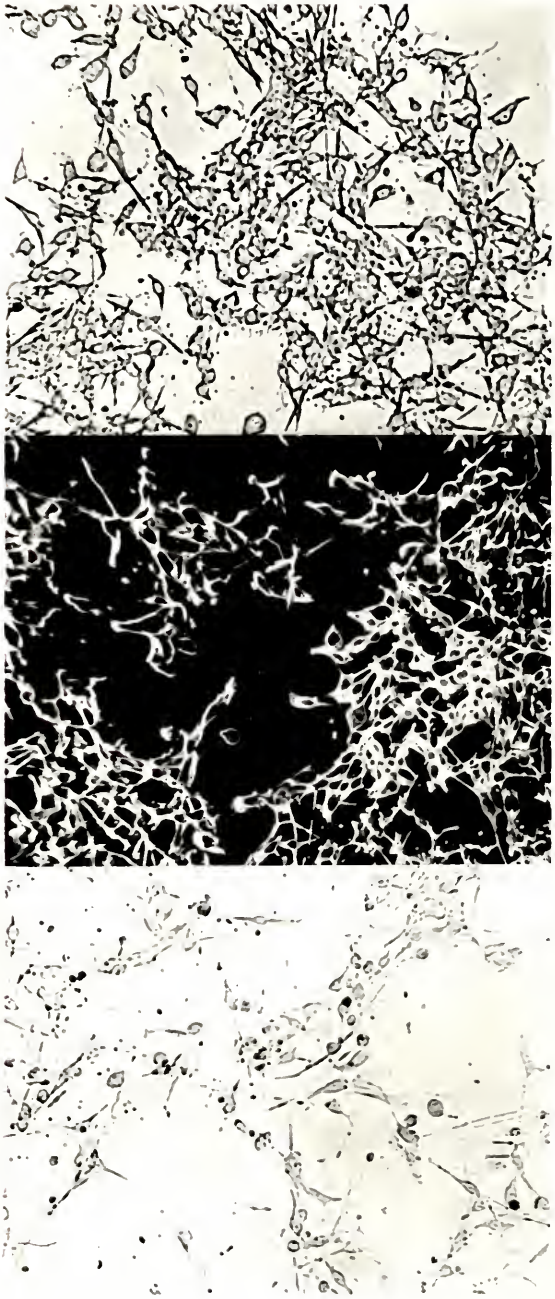

GROUP I
Two weeks
Subcutaneous Tumors
Lungs from Tumor Bearing Mice
Some samples to Belguim
Some samples retained for comparative analysis
Compare (NAA & PIXE)
Control Lungs
All samples sent to Belgium — then returned for comparative analysis (PIXE)
GROUP II
Lungs from Tumor Bearing Mice
Subcutaneous Tumors
Compare (NAA)
Compare (PIXE)
Control Lungs
GROUP III
Compare (NAA)
Tumor (Metastatic) Bearing Lungs
Individual Lung Metastases
Combine
Control Lungs

FIGURE 4. B-16(F10) MELANOMA

Phasephotomicrographs of B-16(F10) melanoma cells (A and B) were taken at 40X power using different phase contrast settings. A photomicrograph (C) of B-16(F10) melanoma cells was also taken using a normal light-inverted microscope. All photomicrographs show the approximate level of confluence in the tissue culture flasks where splitting and resuspension in fresh complete minimum essential media (CMEM) becomes mandatory.


A
B
C

show the cells still firmly attached to the tissue culture flask as noted by the elongated cells. When detached and floating in suspension, the viable cells became spherical. The extent of confluency in the photographs is approximately the maximum to which they were allowed to grow before harvesting. The B-16(F1) subline appeared the same under the microscope, but the growth rate was markedly slower than the B-16(F10) subline.

Normally after 2 to 4 days of growth, each flask of cells was harvested, resuspended in new CMEM, and divided among two to six new flasks, following the steps of Appendix C. Each cycle of harvesting, splitting, and reculturing represented a "passage" for the cells. After nine passages of the initial population of cells, most were frozen for use later.

Cryopreserving B-16 Melanoma Cells

The sequential steps for cyropreservation are contained in Appendix D. In order to assure continuity of the experiment when uninterrupted passages of the cells was not feasible, several vials of cells were frozen and placed in deep-freeze storage. This was also necessary to reduce the risk of altering the biology or trace-element profiles of the original cells. Dr. Fidler pointed out that in successive passages, possibly utilizing CMEM prepared with different batches of fetal calf serum, some trace element concentrations may change in the *in vitro* cell population. This would be a more important consideration if analyses were being conducted directly from *in vitro* cell cultures (Fidler 1979). The cells were frozen in CMEM with approximately 1×10^6 cells per ml. Dimethyl sulfoxide (DMSO) was added to the solution prior to freezing to prevent the cells from lysing when thawed.

Thawing and Reculturing B-16 Melanoma Cells

The materials and procedure for thawing frozen B-16 cells are listed in Appendix E.

Figure 5 is a synoptic schematic of the procedures mentioned above.

Preparation for Injections

The cells were left to grow to confluency and then harvested using 2mM ethylenediamine tetraacetic acid (EDTA). The complete procedure can be found in Appendix F. Cell populations from several flasks were combined to prepare the cell inoculate. There was an average of 1×10^7 cells per flask. The protocol for injections recommended approximately 5×10^4 viable cells in 0.2 ml of Hanks' balanced salt solution (HBSS) for the intravenous injections. By knowing the number of mice to be injected and allowing for losses due to non-viable cells (approximately 10-20%) and a 10% loss ude to waste, the required number of flasks was calculated ahead of time.

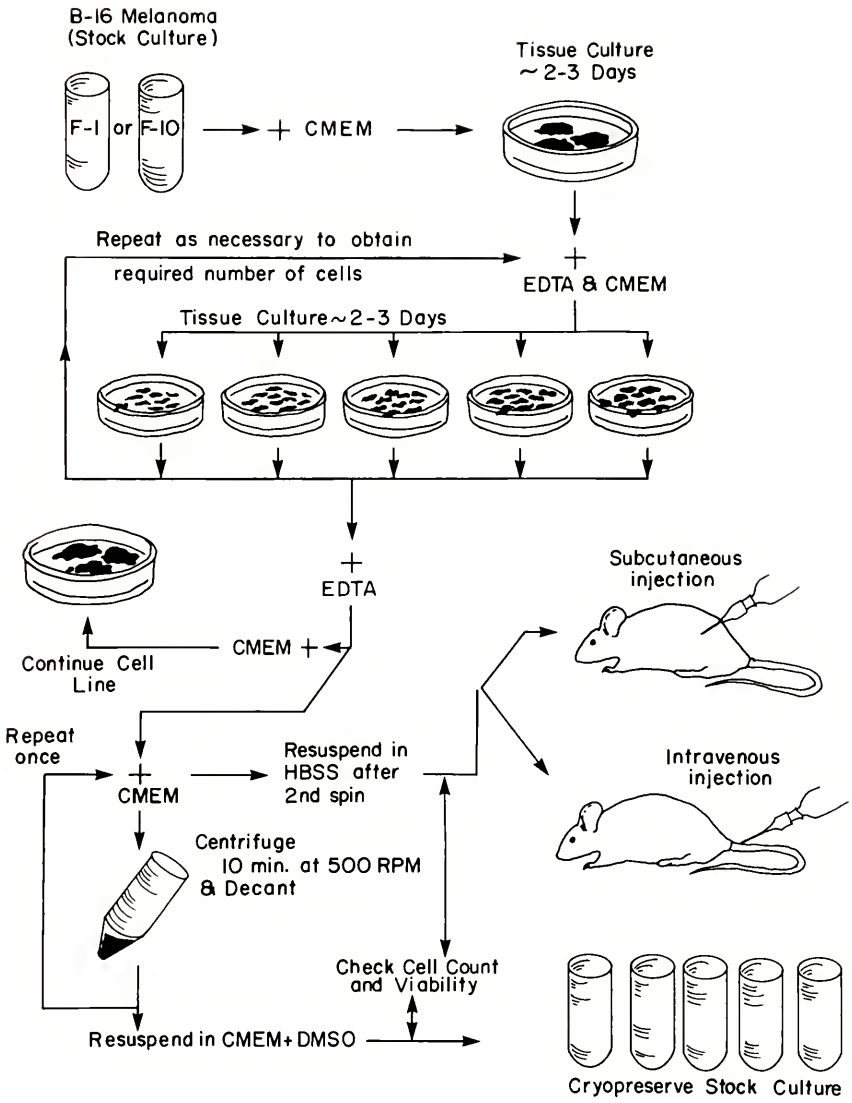
Preparation of Inoculum

The cells were washed twice, once in CMEM and once in HBSS. After each wash they were centrifuged at 500 rpm for 10 minutes. The rpm was found to be critical. Above 500 rpm, the viability of the cells decreased, indicating some type of cell damage. After the second spin, they were again resuspended in HBSS and were assayed for total number of cells with a hemocytometer, and percent of viable cells utilizing the trypan blue exclusion test. The cell suspension was adjusted to a concentration of approximately 5×10^4 viable cells per ml by the addition of HBSS.

Since the cells were in HBSS instead of their essential media, it was important to work quickly to maintain a high percentage of viable cells. Also, Fidler (1975a) reported that B-16 cells have a tendency

FIGURE 5. B-16 CELL CULTURE AND MAINTENANCE

The B-16(F1) and B-16(F10) stock cultures (as received from Dr. Fidler) were suspended in fresh complete minimum essential media (CMEM) and incubated for 2 to 3 days. After each 2 to 3 day period the monolayer of confluent cells attached to the bottom of the tissue culture flask was loosened by overlaying them with ethylenediamine tetraacetic acid (EDTA) for approximately 30 seconds. Each flask of cells was then divided into from one to six new flasks, resuspended in CMEM and incubated. Cells to be used for cryopreservation or injection were removed (as above) from the culture flasks and centrifuged twice with resuspension in CMEM after the first spin only. Cells being prepared for injection were suspended in Hanks' basic salt solution (HBSS) after the second spin while cells being prepared for cryopreservation were resuspended in a mixture of CMEM and dimethyl sulfoxide (DMSO). In both cases, a check of cell count and viability were made prior to injection or freezing.


B-16 Melanoma
(Stock Culture)
F-1 or F-10
+ CMEM
Tissue Culture
~2-3 Days
Repeat as necessary to obtain
required number of cells
+
EDTA & CMEM
Tissue Culture~2-3 Days
+
EDTA
CMEM +
Continue Cell
Line
Repeat
once
+
CMEM
Resuspend in
HBSS after
2nd spin
Centrifuge
10 min. at 500 RPM
& Decant
Check Cell Count
and Viability
Resuspend in CMEM+DMSO
Subcutaneous
injection
Intravenous
injection
Cryopreserve Stock Culture

to clump, and that clumping affects metastatic ability, so it was necessary to keep the cell suspension on ice and vibrate it every 3 to 4 minutes to prevent clumping.

Injection of Animals

The mice were housed in the small animal quarters in the basement of the University of Florida Health Center. This was desirable to insure constancy in environment, diet, and handling. All injections were done in a procedure room designed for such work.

Subcutanteous Injections

The induction of primary tumors was accomplished by injecting tumor cells under the skin. This operation required at least two people and could be speeded up significantly by having a third person to continually load syringes. The injections were made with a 1.0 ml glass, disposable syringe and a 22 gage needle. While the mouse was being securely held, the skin over the right, rear back area was lifted slightly, the needle inserted under the skin parallel to the back, and the inoculum injected slowly (over 3 to 5 seconds) with only slight pressure.

Twenty-two Group I mice received 2.72×10^4 viable B-16(F1) cells (75% total viability) in 0.2 ml of inoculum, while 11 control mice received an injection consisting of HBSS only. Two weeks later, these same Group I mice were inoculated intravenously. The details are discussed below.

Group II mice received subcutaneous injections only. The group consisted of 29 experimental animals and 14 controls. Each mouse in the experimental subgroup was administered 6.02×10^4 viable B-16(F1) cells (80% total viability) in 0.2 ml of inoculum while the controls received HBSS as above.

Intravenous Injections

The prescribed procedure for inducing metastatic lung tumors in the mice was to inject the melanoma cells intravenously into a lateral tail vein. There are two lateral tail veins and either may be used; however, they are very small and some expertise was required to successfully thread the needle into the vein. A 27 gage needle was used for this injection. This procedure was accomplished by placing the animal in a "mouse vise" which held it securely and provided easy access to the tail. To help visualize the veins, they were dilated by heat from high-intensity tensor lamp held close to the tail for 30 to 40 seconds. The syringe was loaded and the needle was bent approximately 20^{o} to aid in threading the vein. It was best to begin at the distal end of the tail for the first injection. The veins run laterally along either side of the tail, so it was necessary to roll the tail 90^{o} in either direction. The needle was inserted at a slight angle into the vein and then rotated so that the needle paralleled the vein. Whenever the needle was in the vein, the inoculum could be injected with very little pressure. However, if resistance was felt, it meant the needle was outside the vein. Usually if the vein had been penetrated, a clot would form and it was impossible to continue with the injection at that location. By attempting another injection proximally (further up the tail toward the mouse) the clot was avoided and, if successful, the tumor cells would then circulate freely.

The Group I experimental mice received 5.00×10^{4} viable B-16(F1) melanoma cells (87% total viability) intravenously two weeks after receiving the subcutaneous injection. The goal was to produce both primary and metastastic tumors within the same animal. The two-week delay between injections was to allow the primary tumor to become established and grow before the pulmonary metastases began.

All Group III mice were given intravenous injections. The experimental subgroup received 6.10×10^4 viable cells (84% total viability) in 0.2 ml of inoculum. All control subgroup mice were injected with HBSS only.

Specimen Collection Technique

The design of the experiment delineated six categories of specimens to be obtained for analysis:

(1) serum from all "experimental"mice,

(2) serum from all control mice,

(3) lung tissue from Groups I and II "experimental" mice,

(4) lung tissue from control mice in all three groups,

(5) primary subcutaneous tumor tissue from Groups I and II mice, and

(6) lung metastases from Group III mice.

At approximately ten days following the subcutaneous injection of B-16(F1) melanoma cells, a palpable tumor was present; however, it was too small to analyze. In the 20 to 30 day post-injection period, very large tumor masses were growing at the site of the injection (Figure 6). At this time, the animals were sacrificed and the specimens collected.

Surgical Procedures

The procedure for collecting the samples are discussed and presented in Figures 7 through 22 which follow.

Sample-Target Preparation

The ultimate goal of PIXE analysis is to determine the elemental composition of a sample. However, because of its relatively high sensitivity (approximately 1×10^{-9} g for most elements of biological interest), combined with the fact that the amount of sample analyzed is about 1 mg, meticulous efforts must be given to insuring that the

FIGURE 6. TUMOR BEARING MOUSE

A C57BL/6j mouse with clearly evident primary tumor at the site of subcutaneous injection on the right flank. This tumor size is typical of 20 to 25 day tumor growth and represents an approximate maximum size that the mouse can support. Mice with tumors of this size or which were showing signs of physical distress were sacrificed.

FIGURE 7. THE ANESTHESIA PROCESS

Since mouse serum was needed for PIXE analysis, it was necessary to anesthetize all animals prior to surgery. This was required in order to obtain the maximum volume of blood during cardiac puncture. A 3 ml syringe holder was used with methoxyflurane soaked gauze as the anesthetic. During the deeper stages of anesthesia, it was necessary to restrain the animal from pushing into the syringe holder and suffocating.

FIGURE 8. INITIAL THORACIC INCISIONS

While the mouse was still partially covered by the syringe holder (B) and in deep anesthesia, a transverse incision was made at the xiphoid process (A). Next an incision was made from the xiphoid process to the trachea taking care not to penetrate the subcutaneous fascia or the thoracic cavity. Clamps were used to retract the skin and maintain a contamination free surface. The final incisions were made through the ribs from the diaphragm to the neck. The xiphoid process was clamped and the rib case retracted toward the head.


A
B

FIGURE 9. THORACIC CAVITY

The retracted rib cage (A) was held in place by a clamp. This caused the heart (B) to extend ventrally and expose the lungs (C). The ventral extension of the heart simplified the cardiac puncture process. At this point, the lungs had collapsed and respiratory attempts were sporadic.


A
B
C
C

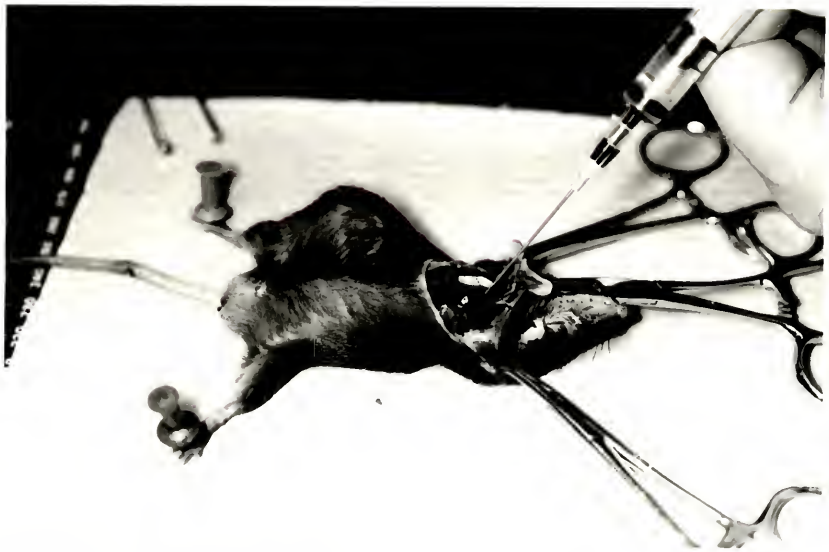


FIGURE 10. CARDIAC PUNCTURE

Cardiac puncture must follow the initial incision within 0.5 to 1.0 minute. The puncture was made using a 22 gage 1.5 inch needle with a 3 ml disposable syringe. Larger needles (20 gage) were evaluated but caused excessive cardiac bleeding. Smaller needles tended to become blocked. Only very slight negative pressure was used in order that the cardiac chamber could remain full by its own pumping action. Increased negative pressure quickly caused wall collapse and needle blockage. It was sometimes helpful to rotate the needle slowly while holding the plunger stationary. This intended to reduce needle blockage. Cardiac fibrillation usually followed the initial puncture in about 1 minute. Blood volume obtained ranged from 0.5 to 2.5 ml. Larger blood volumes were obtained from tumor bearing mice due to their increased blood supply and reduced clotting capability. The needle was then removed from the syringe and the blood slowly transferred to a sample container. Rapid transfer of the whole body or expulsion through the needle caused hemolysis with consequent iron contamination of the serum.

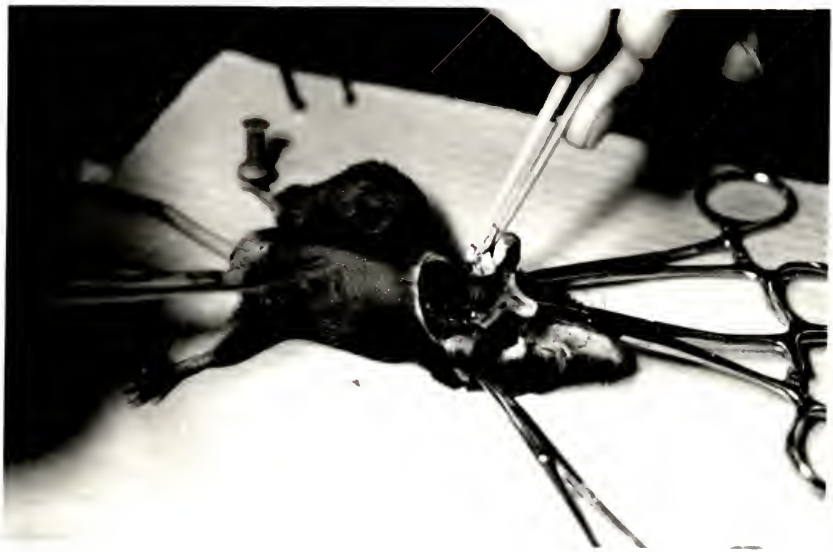


FIGURE 11. LUNG REMOVAL

Following cardiac puncture the lungs were excised. Care was taken to use Teflon coated tweezers during the process in order to reduce metal contamination. In all cases the lungs were placed in sample vials containing 5% dextrose in water. This solution was also later used as a primary wash for all samples. Dextrose (5% in water) was found to be suitable due to its isontonic nature and lack of sodium and other contaminating materials.

FIGURE 12. CONTROL LUNGS

Lungs removed from control mice. Washing was performed in sterile plastic petri dishes. Surface contamination consisting of hair, blood clots, and blood was removed with 5% dextrose. A final quick rinse using double distilled water was used to remove as much of the dextrose solution as feasible. Samples were then immediately placed in polyethylene containers and frozen in liquid nitrogen in preparation for lyophilization.

FIGURE 13. METASTATIC LUNG TUMORS

Pictured are lungs removed from Group III mice. Group III mice received an intravenous injection of B-16(F10) melanoma cells. These lungs were removed 31 and 33 days post injection. Multiple metastases are clearly visible. Washing and sample preparation paralleled that of control lungs with the exception that large single metastatic tumors were removed for PIXE analysis independent of lung tissue. INAA analysis was conducted on combined groups of lungs as shown here.

FIGURE 14. METASTATIC/CONTROL LUNG COMPARISON

Lungs removed from a Group III mouse with multiple lung metastases (A) are shown with those from a control mouse (B). All metastatic lungs were larger and more massive than control lungs. Amelanotic spherical bulges in the lung (A) could be nonpigmented tumor tissue or, more probably, distended lung parenchyma.

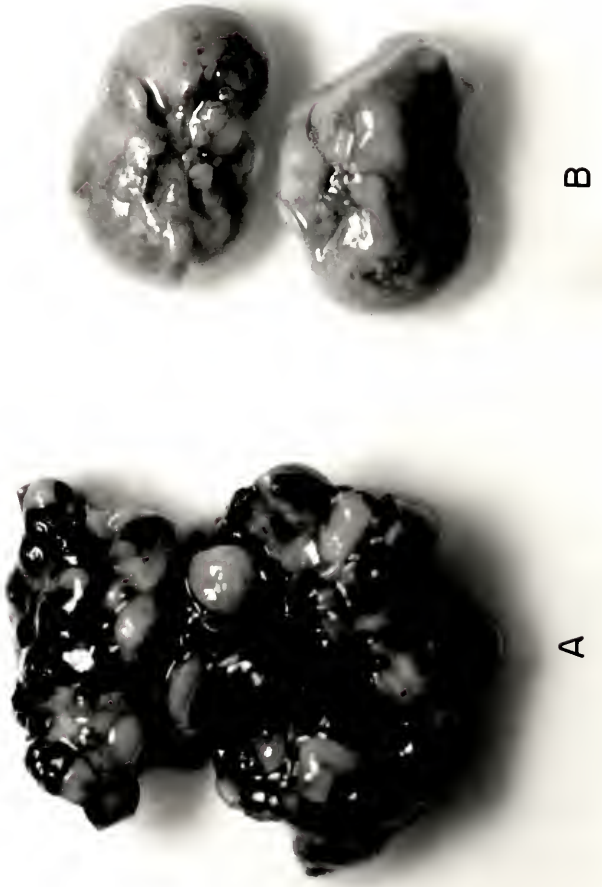

B
A

FIGURE 15. 11-DAY SUBCUTANEOUS TUMOR

This tumor represents a typical size for 11 days post injection. It grew on the basal layer of the skin at the site of injection.

FIGURE 16. 11-DAY SUBCUTANEOUS TUMOR (EXPANDED FIELD)

This is a close-up photograph of the previous figure, more clearly showing the tumor and its associated surrounding vasculature. Also evident is the hair contamination from the incision. For this reason, the surface of each sample was quickly washed with 5% dextrose solution after excision.

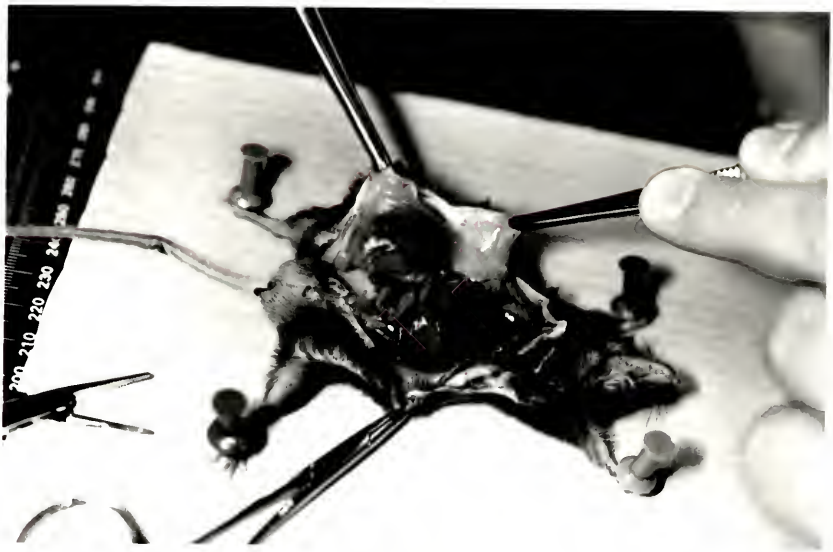


FIGURE 17. 23-DAY SUBCUTANEOUS TUMOR

A relatively large 23-day (post subcutaneous injection) tumor was being prepared for excision. The xiphoid process was extended ventrally and the heart and lungs had been removed. An abdominal incision was made, at midline, from the diaphragm to the tumor side of the testicular area. The clear film over the tumor area was the peritioneum which was usually left intact to contain the abdominal organs. For this reason, the midline incision was done with surgical scissors inserted between the superficial fascia and the peritoneum. When this was done correctly the abdominal organs were contained and did not interfere with tumor removal. After this incision, the skin was retracted with clamps while a scalpel was used to separate the superficial fascia from the surface of the tumor. The tumor was excised when it had been isolated from the basal layer of the skin to the maximum extent possible.

FIGURE 18. 23-DAY SUBCUTANEOUS TUMOR (INVASIVE)

A relatively small 23-day (post subcutaneous injection) tumor had invaded the femur and its associated musculature. The peritoneum (held by a clamp in the lower center of the photograph) is clearly evident. Tumors which had invaded bone and muscle were more difficult to excise and care was taken to prevent calcium contamination from bone chips.

FIGURE 19. 33-DAY SUBCUTANEOUS TUMOR

This shows a typical size for a 33-day (post subcutaneous injection) tumor. It had invaded the femoral area and the peritoneal cavity. It was doubtful that this animal could have lived another 24 hours.

FIGURE 20. 33-DAY SUBCUTANEOUS TUMOR (EXPANDED FIELD)

This is a close-up view of the previous figure. Due to the invasiveness of this tumor, the peritoneum ruptured exposing the intestines. The hole in the skin at the top of the photograph was caused by cannibalistic attacks by other mice. These attacks were fairly common.

FIGURE 21. TUMOR EXCISION

This photograph shows the excision of the tumor shown in the previous figure. Immediately after excision, while still held by forceps, the tumor was quickly rinsed with 5% dextrose.

FIGURE 22. SUBCUTANEOUS TUMOR (EXCISED)

Most 29- to 33-day (post subcutaneous injection) tumors had a volume of 4 to 8 ml. Lyophilized weight ranged from 350 to 1000 mg and wet weight was in the 4 to 8 g range. The tumor pictured here was shown in the two previous figures.


CENTIMETERS
1 2 3 4 5 6 7 8
4 8 12 16 20 24 28 4 8 12 16 20 24 28 4 8 12 16
INCHES 1 2 3

"target" is truly representative of the specimen. Two factors which must be kept in mind constantly are (1) prevention of contamination and (2) homogeniety of the sample.

Sample handling and target preparation, while obviously related, are two very distinct operations. For biological samples, the handling procedure must be performed within a few hours after collection, but the preparation of targets can be done days or even months later. Step-by-step guidance on the handling of tissue specimens used in this research and the preparation of sample targets are contained in Appendices G and H, respectively.

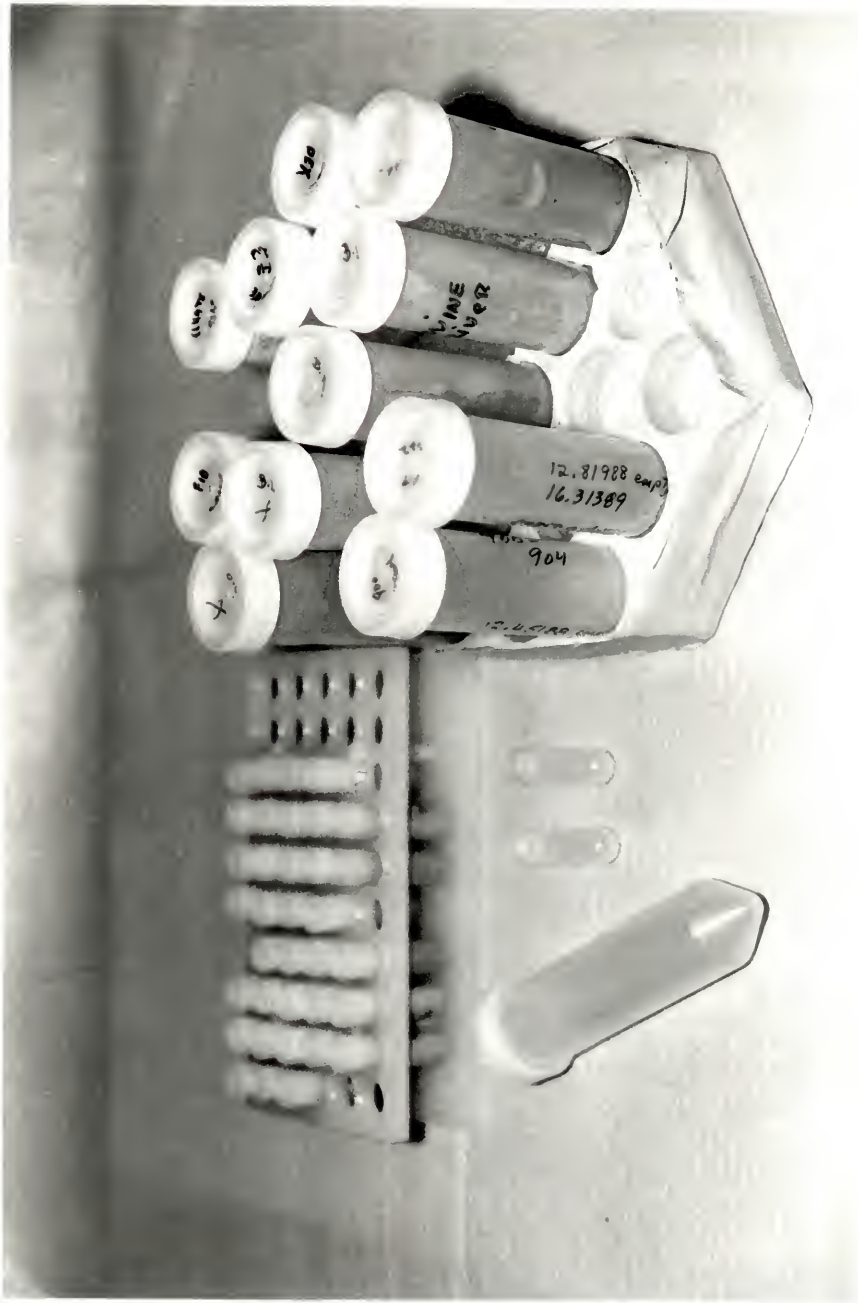
Tissue Samples

Tissue samples consisted of primary subcutaneous tumor, lung metastases, and control lungs. All tissues were rinsed with 5% dextrose solution immediately after excision and placed in clean, sterile polyethylene containers. The lung tissues, including the individual metastases, were covered with 5% dextrose solution. Although the surgery was performed at the Medical Center, all specimens were brought to the Nuclear Sciences Center for further handling.

All specimens were given another rinse with the dextrose solution to remove any loose tissue, blood, or contaminants. A final, thorough wash was given to each sample with double-distilled, deionized water. These procedures were meticulously carried out in clean, sterile petri dishes for each sample, using only Teflon (Dupont) coated instruments. Following the last wash, the samples were placed into polyethylene centrifuge tubes (Figure 23) and deep-frozen in liquid nitrogen. The subcutaneous tumors were large enough that individual samples could be split and analyzed by both PIXE and INAA. However, due to the little

FIGURE 23. SAMPLE CONTAINERS

The fresh tissue samples were stored in the large (50 ml) centrifuge tubes during lyophilization. The same containers were used to pulverize the samples by brittle fracture. The powdered material was transferred to the small (2 ml) polyethylene vials for subsequent spiking and storage.


SWINE
LIVER
12.81988
16.31389
904

amount of material from the single largest metastasis in the Group III mice, it was decided that they would be analyzed solely by PIXE, and the remainder of highly metastasized lung would be analyzed by INAA. The lungs from control mice were too small to analyze (individually); therefore, depending on their size, anywhere from three to eight lungs were combined into a single sample. By doing this, analyses by both techniques could be accomplished on the same sample.

The centrifuge tubes were covered with filter paper (held on by rubber bands) and placed in a lyophilizer for 24 to 48 hours (Figure 24). All samples were completely dehydrated in that time. This left a solid, fibrous material, quite reduced in bulk, which was difficult to separate. In order to homogenize the samples, the centrifuge tubes were placed in a styrofoam holder which was filled with liquid nitrogen. After 1 to 2 minutes, the samples became frozen and brittle. It was then possible, using a high purity quartz rod for a pestle, to pulverize the tissue into a powder, similar to the technique described by Iyengar and Kasperek (1977). The sample was extremely dry and the fine powder, produced in the grinding, readily became airborne, so a surgical mask was worn during the procedure to prevent inhalation of the powder.

After the samples were pulverized, they were again lyophilized for 24 hours to remove any moisture which was absorbed during the grinding process. Next the powder was divided for the separate analyses. The portion for PIXE analysis was transferred, via a Teflon coated spatula, into preweighed and labelled polyethylene vials which had a tight-fitting screw cap. The transfers were done quickly with less than 30 seconds exposure of the powder to the air. Each vial was then reweighed to determine the quantity of sample it contained.

FIGURE 24. LYOPHILIZATION OF SAMPLES

Prior to division of the samples for PIXE and INAA analysis, all samples were lyophilized (freeze-dried) at least twice. The first lyophilization usually took place within several hours of the surgical procedure. Each subcutaneous tumor was placed in a labelled centrifuge tube. Enough lungs and serum to insure adquate sample size were placed in other labelled centrifuged tubes. Depending upon the size of the lungs or the blood volume from individual mice, from three to eight mice were required to provide enough material. After the initial lyophilization, samples were homogenized using a "brittle fracture" technique, and relyophilized to remove water absorbed during the process.

This figure shows a rack of centrifuge tubes being placed in the lyophilizer, which is simply a vacuum apparatus capable of trapping water withdrawn from cryogenically frozen samples.

Blood Samples

Immediately after the blood was drawn from the mice, it was transferred into sterile, plastic test tubes and capped. Since the amount of blood collected from each animal was so small, it was necessary to combine three to four samples into one tube. The blood was then spun on a microcentrifuge at approximately 3000 rpm for 10 minutes to separate out the serum. The serum was transferred to new test tubes, covered with filter paper, and frozen until placed in the lyophilizer for 48 hours. Following the freeze-drying process, a pale-yellow crystalline substance remained.

Spiking the Samples

The amount of sample material actually irradiated in the beam during PIXE analysis is unknown and may vary considerably from one target to another. Because of this, it is impossible to quantitate the elemental concentrations based solely on the mass of the target. A well-established method for normalizing, or standardizing, analytes in spectrochemical analysis is by internal standardization (Bertin 1978). In this method, a known quantity of an element is added (spiked) to the sample and its concentration can be calculated. The internal standardization method was used in this research. The dopant chosen was ultra-pure silver nitrate, due to the fact that it is readily soluble in water and the silver K-lines do not interfere with the x-ray lines of the biologically important elements. To reduce the weighing error, it was best to weigh 100 to 200 mg of sample rather than small quantities of 10 mg or less. Also, the spiking of 100 to 200 mg quantities of sample produced a lower spiking error than the spiking of 5 to 10 mg of sample. Since the amount of material to be deposited on a target was on the order of 1 mg,

it was not only important that the sample be homogeneous, but also that the dopant be uniformly distributed throughout the sample. Because the powdered tissue was exceedingly dry and not soluble in water, it was not feasible to add the spiking solution directly. First, the samples had to be moistened slightly so that the dopant could spread freely throughout the powder. Double-distilled deionized water was added to the sample (using a 50 µl automatic micropipetor) in the ratio of 2 to 3 µl per mg of sample (see Appendix G for an example). This was added in 50 µl aliquots with sufficient time between "shots" for the water to soak into the sample. This would often take up to 3 to 4 hours. If all the water was added at once, the powder would float up and adhere to the sides of the vial. After the water was "soaked in," the vials were placed in a 35°C oven for 2 hours to dry slightly, before the silver dopant was added. The stock solution was made in a concentration of 2.670 µg of silver per µl of solution.[1] The desired silver concentration was approximately 3000 ppm. This could usually be added at one time because the surface tension was broken due to the dampness of the sample and the dopant would readily soak into the sample. The samples were then placed in a 60°C oven until the solution was distributed throughout the material, then the temperature was raised to 80°C for 48 hours. After that, the vials were tightly capped until preparation of targets.

Target Preparation

This was the point at which contamination control became vitally important. All target preparation was done in a filtered-air hood.

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[1] 1.21372 g $AgNO_3$ in 250 ml of double-distilled, deionized water. Manufacturer's quantitative analysis: 61.8% Ag per unit of $AgNO_3$. Spex Industries, Inc., Metuchen, NJ.

There are essentially four components of a target: (1) the target ring or frame, (2) the backing material, (3) the sample material, and (4) the Formvar covering.

Target ring

The purpose of the target ring was to provide a frame upon which to mount the backing material. Since the sample was in a powdered form, some device was necessary to be able to secure the backing material in position for proton bombardment. A polyethylene ring, 2.5 cm in diameter,was chosen because of its strength, durability, and low Z (atomic number) material.

Backing material

The backing material was necessary to hold the sample in place during irradiation. The choice of a backing material is extremely important, not only because it must withstand bombardment of the proton beam; but, also, it must be relatively free of high-Z contaminants which may interfere with the sample spectrum. Additionally, the backing must be thin so that the x-ray background will be low. Aluminized Mylar (Dupont),[1] selected for the backing in this experiment, was 0.7 mg per cm^2 in thickness and contained approximately 10 µg of aluminum per cm^2. In some previous PIXE analyses, it has been reported that charge build-up has occasionally been observed if the protons stopped in the target do not have a path to ground. Charging interferes with the analyses by increasing the background, thus decreasing the sensitivity. Maenhaut et al. (1980) reported the charging problem was completely eliminated by the use of aluminized Mylar.

[1] Furnished by Dr. Willy Maenhaut, Institute voor Nucleaire, Wetenschappen Rijksuniversiteit Gent, Gent, Belgium.

The backing material came on a roll, in two layers with a protective paper covering each layer. The aluminum was deposited on only one side of each Mylar strip and was readily identified upon inspection. Complete backings were made up in advance and stored in sterile, disposable petri dishes. The Mylar was stretched out on a glass cover which had been thoroughly cleaned with alcohol. Because of the size of the glass, it was convenient to make five backings at a time (see Figure 25). With the aluminized side of the Mylar facing down, the five polyethylene rings were lightly sprayed with an adhesive[1] (outside the hood) then placed on the Mylar with forceps. A scalpel was used to trim the Mylar around the edge of the rings which were then transferred to petri dishes and covered until the sample material was to be added (see Figure 26). Generally, three to five targets were prepared from each sample and stored in petri dishes.

Sample Material

Tissue. The ideal form for the sample material to make thin, uniform targets, was a fine, homogeneous powder, but when the spiked material came out of the drying oven, it was in the form of a very hard pebble. To achieve a homogeneous powder, the materials were ground in an agate mortar and pestle (Figure 27). Because the sample was so hard, it had to be covered by filter paper and the pestle crushed the material from on top of the paper--otherwise, pieces of material would shoot out of the mortar. Once the material was broken into smaller pieces, the filter paper could be discarded and grinding would progress

[1] 3M Company, Photo Mount Spray Adhesive.

FIGURE 25. PREPARATION OF TARGET BACKINGS

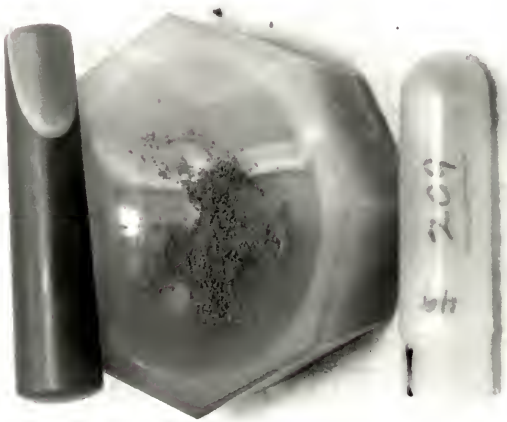
Aluminized Mylar was used to support the sample material during PIXE analysis. Polyethylene mounting rings were glued onto the Mylar strip and a scalpel blade was used to trim around the edges.

FIGURE 26. STORAGE OF SAMPLE TARGETS

As the target backings were prepared, they were stored in plastic, disposable petri dishes. The finished targets were also stored and transported in petri dishes.

FIGURE 27. AGATE MORTAR AND PESTLE

A prime requisite for PIXE analysis is that the sample be homogenous. Prior to placing the sample material on the backing, it was thoroughly ground and mixed in an agate mortar. The homogenized powder was stored in polyethylene vials as shown.



normally. It was important that the grinding be thorough and the mixing complete to insure homogeniety of the material.

When the sample was pulverized, it was ready to transfer to the backings. A petri dish of previously prepared backings was selected and the cover was labelled to correspond to the sample number. To make the targets, the rings were placed on top of a rubber stopper (aluminized side up) being careful to handle them only by the edges (Figure 28). With a Teflon coated spatula, approximately 1 mg of sample was distributed in a 6 mm diameter circle in the middle of the backing.

Serum. The lyophilized serum was resuspended in double-distilled, deionized water prior to spiking. Each dried sample was transferred into new, preweighed polyethylene vials and the net weight of the material measured. Initially, water was combined with the serum in the ratio of 10 µl per mg; however, most suspensions were extremely viscous and not completely dissolved. More water was added, but for some unknown reason, most samples did not dissolve and had to be discarded. Five control and five experimental mice serums resuspended satisfactorily and were doped with the stock silver-nitrate solution in a ratio of approximately 2 µl per mg (dry weight) of serum. The exact concentrations were then calculated.

Since the samples were in liquid form, 20 µl was pipetted directly onto each backing and they were then placed in the vacuum dessicator for 1 hour. As they were dehydrated, some of the material curled up on the edges and separated from the Mylar.

FIGURE 28. TARGET PREPARATION

The Mylar backings were supported on a rubber stopper, then the sample (approximately 1 mg of material) was distributed in a 6 mm diameter circle in the middle of the Mylar. The targets were prepared in a hood with positive-pressure filtered-air in the work area. All contact with the sample was with plastic coated instruments to avoid metal contamination.

Formvar[1] covering

Tissue. Since the sample was a dry powder and the target had to be mounted vertically during irradiation, the material had to be fixed to the Mylar backing. Fixation was accomplished by covering the powder with a slightly wet, thin Formvar film. A 1% solution of Formar was prepared by dissolving 1 g of the resin in 100 ml of 1, 2-dichloroethane. This thin film was made by dropping 10 µl of the solution (with an automatic micropipetter) onto the surface of double-distilled, deionized water. After about 20 seconds, the film was lifted off of the water (Figure 29) with a thin aluminum plate. The film could be seen on the surface of the water and by carefully maneuvering the plate under the film and lifting slowly, it was removed intact. The aluminum plate had a hole cut out slightly larger than the target rings. Before actually placing the Formvar on the target, the scalpel was used to cut a hole along one edge of the film, about 5 mm from the edge. The film was placed on the target so that one portion of the aluminized Mylar was not covered by the Formvar. This was done so that when the target was mounted in the holder, the aluminized surface would not be insulated from the holder by the film. The aluminum plate was passed downward over the target until the film separated at the edge of the ring (Figure 30).

After the three targets were prepared and fixed, they were transferred, in the covered petri dish, into a vacuum dessicator. The valve to the vacuum pump was opened slightly, allowing the air to escape slowly, so that any air trapped under the film could escape and make a tight seal with the Mylar. After 20 to 30 minutes in the dessicator, the targets were ready for analysis.

[1] Polysciences, Inc., Rydal, PA

FIGURE 29. THE FORMVAR FILM

To fix the sample material onto the Mylar backing, a plastic film was placed over the target. The thin film was made by placing 10 µl of the Formvar film on the surface of water. It could be picked up by maneuvering an aluminum plate underneath and lifting carefully.

FIGURE 30. COVERING THE TARGET WITH FORMVAR

The thin film was positioned over the target and passed downward until the film separated at the edges. The targets were then placed in a vacuum dessicator which allowed air trapped between the Mylar and film to escape and form a smooth seal.

Serum. Although the serum samples adhered to the Mylar backings when dried, some of the material curled up and appeared that it might separate. Therefore, a Formvar film was placed over these samples also, but the procedure was altered slightly. As soon as the film spread out on the surface of the water, it was picked up and placed on the serum. It was still quite wet, but the water soaked into the serum and caused it to lay back down flat on the Mylar. The targets were then replaced on their labelled petri dishes, covered and placed in the vacuum, under low suction, for 30 minutes.

Preparation of Reference Standards

Four standards were used to establish the calibration curve for quantitative analysis: (1) bovine liver, (2) orchard leaves, (3) Bowen's Kale, and (4) thin film standards.

Bovine Liver, Orchard Leaves and Bowen's Kale

The first two standards were prepared by the National Bureau of Standards and the third (a reference material only) by H.J.M. Bowen of Berkshire, England. They were used to calibrate and verify the system for analyses of elemental concentrations. They are discussed in more detail later.

The handling and target preparation were no different for these standards than for the tissue samples just described. All three reference materials were in the form of a very fine powder. Approximately 400 mg of each material was transferred into clean, polyethylene centrifuge tubes, immersed in liquid nitrogen for 5 minutes, then placed in the lyophilizer for 24 hours. Next, they were transferred into 2 ml polyethylene vials and tightly capped to prevent absorption of moisture from the air. The empty weight of the vials had

previously been determined, so the sample vials were again weighed to measure the amout of sample contained therein. The samples were then moistened with bidistilled water and spiked with the silver-nitrate solution. The oven drying procedure and target preparation followed the same steps as described above for fresh tissue samples.

Thin films

Thin films especially prepared for PIXE analysis were run to verify the calibration of the system and to determine its sensitivity. The films were cut in individual disks the size of the mounting rings; therefore, the only preparation involved spraying the adhesive on the rings and mounting the films.

Storage, Handling, and Transport of Targets

The targets were kept in covered petri dishes at all times when out of the hood. Since several petri dishes of targets could fit into the dessicator, they were stored and transported to the Van de Graaff accelerator in the dessicator, and removed only when a particular sample was to be analyzed. Forceps were used to lift the targets out of the petri dishes and set them in the target holder. Sample targets ready for analysis are shown in Figure 31.

PIXE Set-up

A beam of 2.5 MeV protons, used in this research, was provided by a Van de Graaff accelerator, Model K-4000, manufactured by High Voltage Engineering Corporation. The beam was focussed by a quadrapole magnet, diffused by a gold foil, and defined by a tantalum and two graphite collimators. The final collimator had an aperture of 5 mm. From the perspective of Figure 32, the protons travelled from right to left inside

FIGURE 31. TYPICAL PIXE TARGETS

Up to five targets could be mounted on the "ladder" at one time. This assembly fit vertically into the irradiation chamber and the targets were lowered into position for analysis sequentially. The PIXE analysis, the data acquisition is done at the time the garets are being bombarded. The samples do not become radioactive, as in the INAA technique.

METRIC
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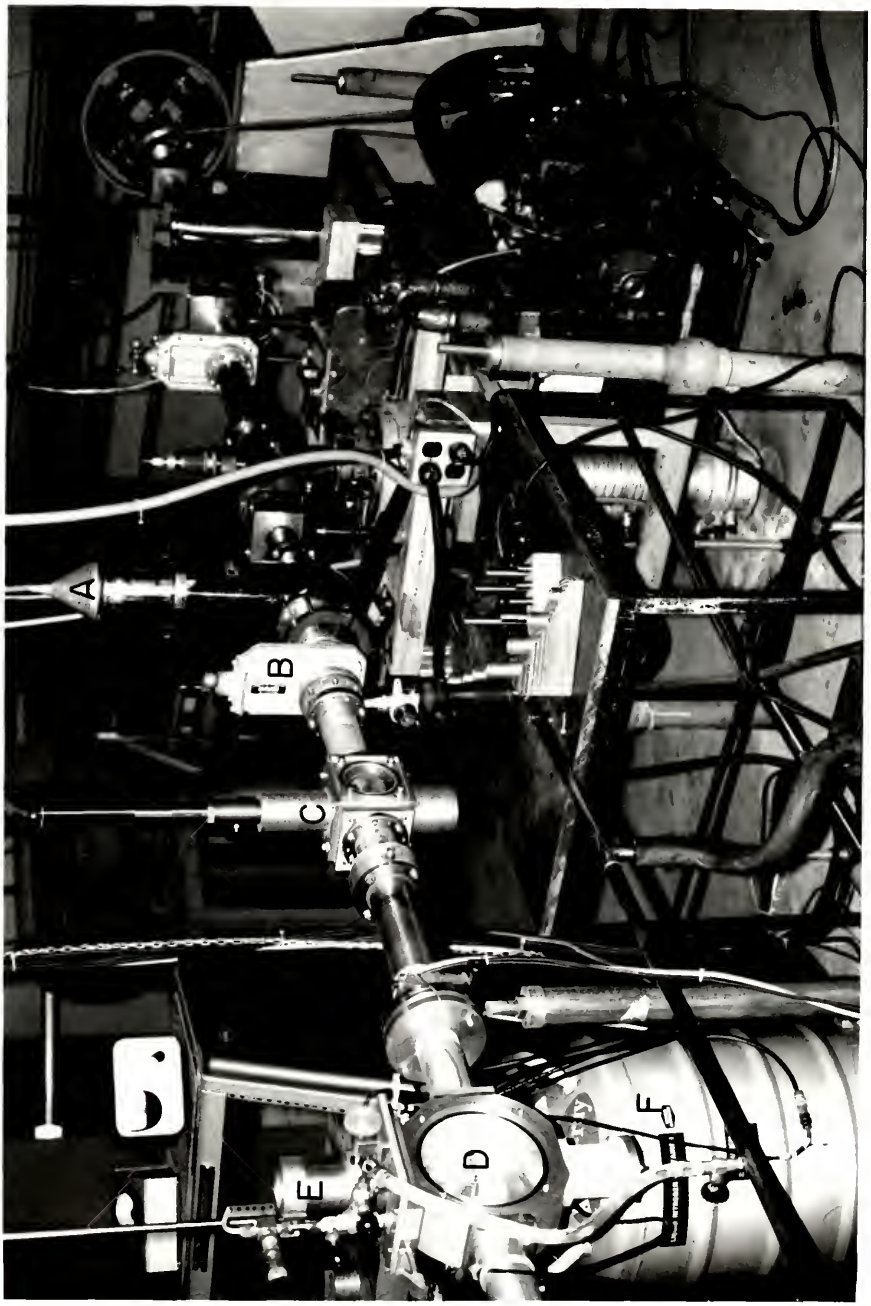
19

FIGURE 32. EXPERIMENTAL ARRANGEMENT FOR PIXE ANALYSIS

The proton beam passed from right to left through the horizontal beam tube, which was kept at a high vacuum by a turbomolecular pump. A liquid nitrogen-filled cold trap (A) assisted in maintaining the vacuum by condensing any gases remaining in the system. When target barrels were exchanged or maintenance was performed on the irradiation chamber (D), the blade valve (B) was closed to hold the vacuum on the accelerator end of the system. Gold diffuser foils were arranged on a moveable ladder in the chamber (C), up line from the irradiation chamber. A manual vacuum interlock antechamber (E) was necessary to maintain the vacuum on the beam line while exchanging target barrels. The Si(Li) x-ray detector was cooled by a coldfinger which was kept in a dewar (F) of liquid nitrogen.


A
B
C
D
E
F

the highly-evacuated (1×10^{6} Torr) horizontal tube. A cold trap (A) was filled with liquid nitrogen during data acquisition to condense any gases out of the system. Sample targets were lowered into the irradiation chamber (D) through a vacuum interlock system (E) which kept the detector and beam line under vacuum while exchanging target holders. On those occasions when the "experiment" end of the line had to be raised to atmospheric pressure, such as for maintenance or repair, a blade valve (B) was closed to maintain the vacuum on the "accelerator" end of the apparatus. Quartz disks were spaced at various points along the beam tube, including one in the diffuser foil chamber (C), to aid in aligning and centering the beam. The interaction of the protons with the quartz produced a bright-blue glow. During irradiation of the targets, the same phenomenon occurred on the aluminized Mylar backing material which made it possible to center the sample in the beam. The target could be observed through a plastic window and adjustments could be made to move the targets either vertically or in a transverse direction in the chamber (Figure 33). The target was positioned at an angle of 67.5° with respect to the beam and the detector was situated, in the horizontal plane, at an angle of 315° with respect to the beam (Figure 34).

Because x rays are emitted isotropically from the sample during irradiation, there are many scattered throughout the chamber. A scattered x ray is degraded in energy, and therefore, loses its identity as a "characteristic" x ray; that means it is no longer useful for identifying the specific element from which it arose. If scattered x rays enter the detector, they only contribute to the background in the spectrum and thus, reduce the sensitivity of the system. To reduce the number of scattered photons which were detected, a high-purity aluminum collimator

FIGURE 33. IRRADIATION CHAMBER

O-rings provided a seal to maintain the high vacuum during irradiation, but the side of the chamber could easily be removed for changing collimators or absorbers. The plastic window provided a view of the targets during irradiation.

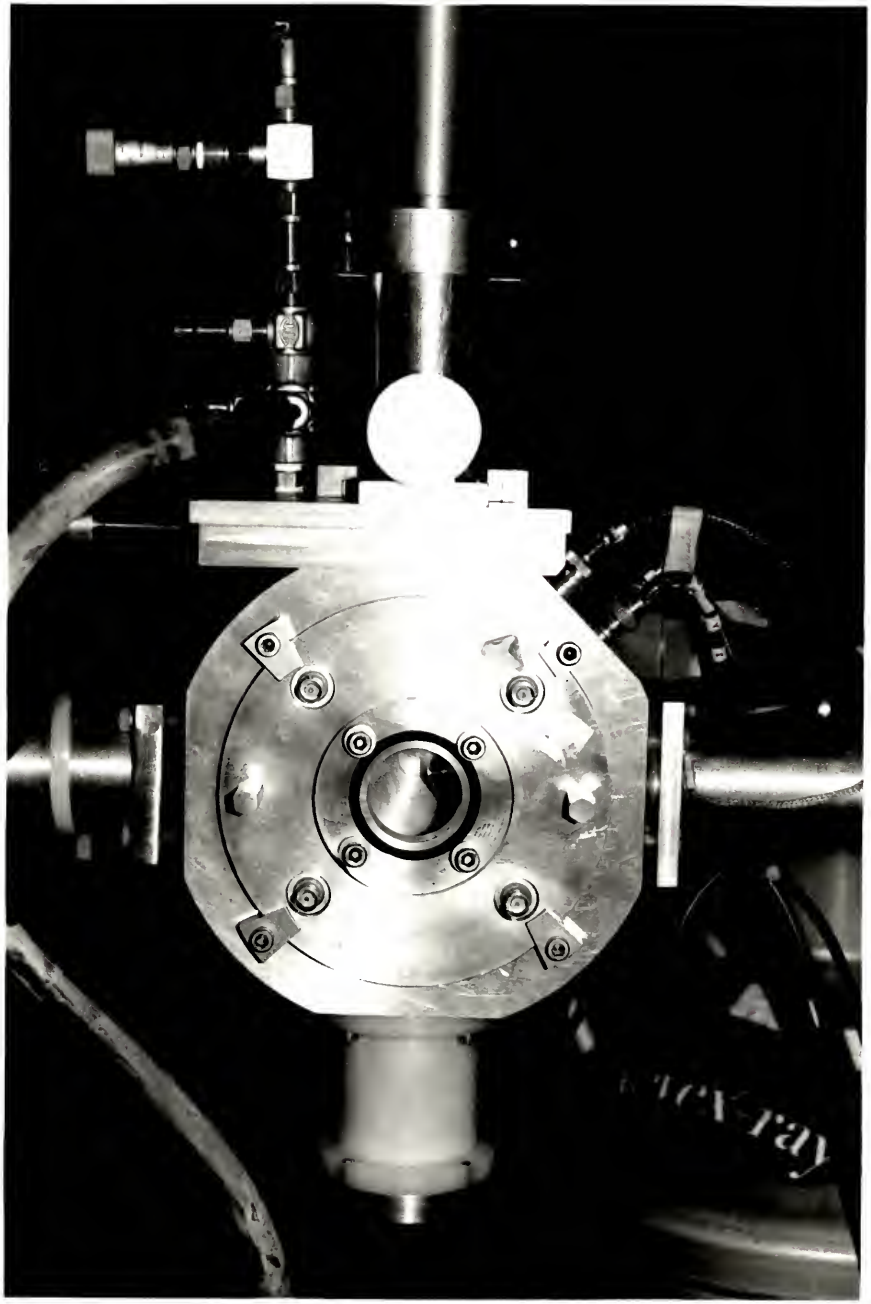

X-ray

FIGURE 34. IRRADIATION CHAMBER (SIDE REMOVED)

The final graphite collimator (A), defined the proton beam to a 5 mm diameter on the target. A sample is shown in position for irradiation. To reduce the number of scattered x rays entering the detector, a high-purity aluminum collimator (B) limited the field of view of the detector to the irradiated portion of the target.


A
B

and a Mylar absorber were placed between the sample and the detector as shown in Figure 34. The 3.7 cm long cylindrical collimator had an aperture of 6 mm and held a 0.71 mm thick Mylar absorber.

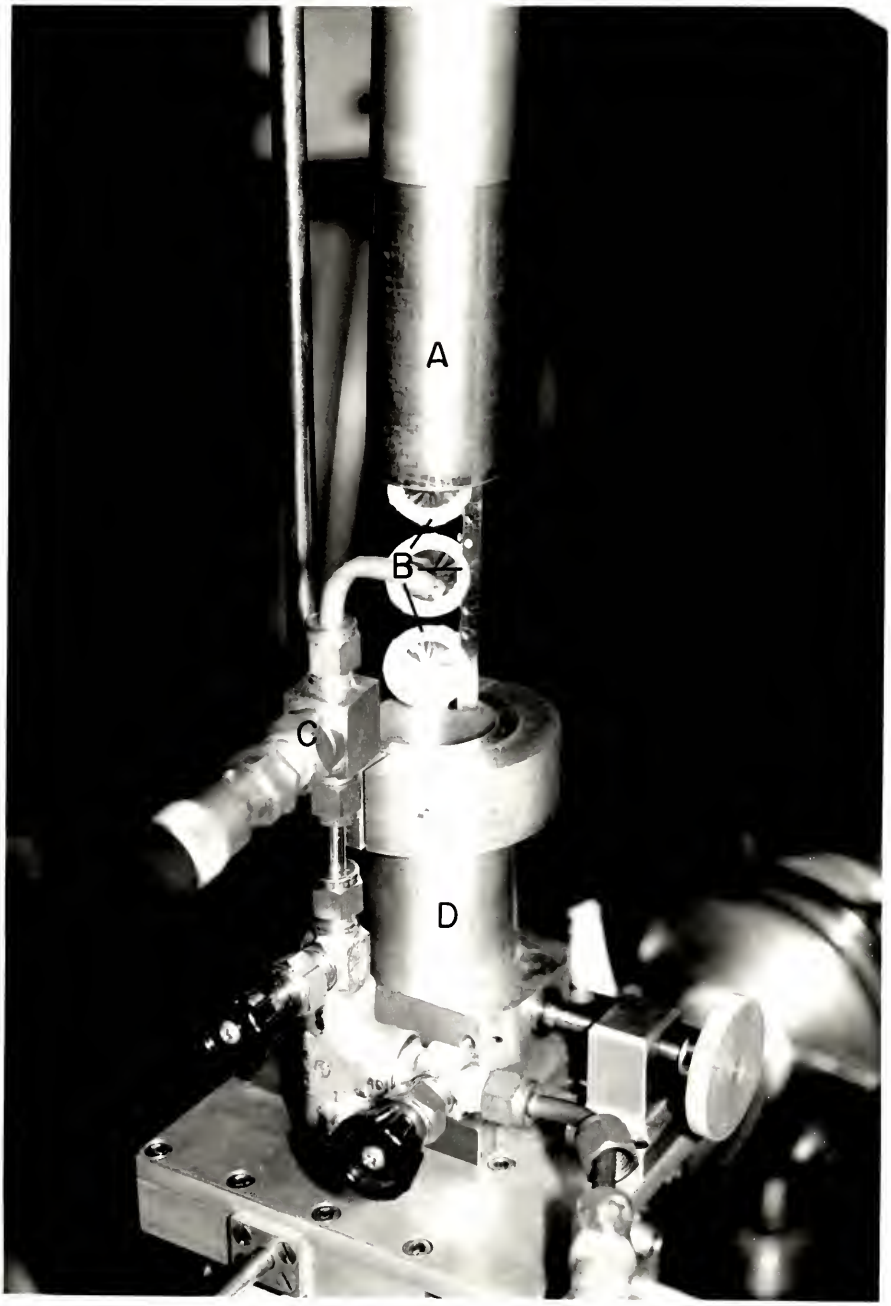
Figure 35 shows a close-up view of a target barrel being lowered into the vacuum interlock system. The needlevalve (C) regulated the rate at which air entered or was pumped out of the chamber, so that neither the targets nor the detector window was damaged by sudden changes in air pressure.

A KEVEX Si(Li) detector was introduced into the irradiation chamber by means of a side port with a vacuum seal provided by an O-ring around the end cap of the detector. The detector's active volume is 30 mm^2 by 3 mm (per manufacturer's specifications) and it has an energy resolution of 158 eV (FWHM), as measured for the 5.89 KeV manganese Kα x ray. The detector and its preamplifier were further amplified and shaped by a KEVEX Model 4525 P Pulse Processor/Amplifier. It contained a pulse pile-up rejector and live-time correction circuit to account for the rejected pulses.

X-ray spectra were accumulated by an LSI-11 (Digital Equipment Corporation) based multichannel analyzer manufactured by Tracor Northern (Model 1710). All data were stored on floppy disks for later analysis. The beam current through the targets was normally kept at 70 to 90 nA and spectra were usually acquired for a preset charge of 140 μC. The time required to accumulate a typical spectrum was 20 to 25 minutes.

FIGURE 35. DETAILED VIEW OF THE VACUUM INTERLOCK

The target barrel (A) was designed to hold up to five targets (B) in a "ladder" arrangement. After the needlevalve (C) closed, an O-ring on the bottom of the barrel provided a vacuum seal. The antechamber (D) was evacuated by a separate vacuum pump before being opened to the irradiation chamber.


A
B
C
D

Spectrum Analysis

Quantitative analysis of trace element concentrations utilized the internal-standard method as mentioned previously. All sample materials, including those from the National Bureau of Standards (NBS), were spiked with silver nitrate prior to irradiation. Since the amount of an element present in a sample is proportional to the area of its associated peak on a pulse-height distribution, its concentration can be measured by its net area in the spectrum.

Calibration

Two NBS standard reference materials, bovine liver and orchard leaves, were the basis for the calibration curve. Each contains a variety of elements whose concentrations are certified by NBS. Also, samples of Bowen's kale powder, which does not have certified elemental concentrations, but for which ranges of concentrations have been reported in the literature, were analyzed for corroboration. The advantage of using these materials for calibration is that they, being biological in origin, are similar to the specimens obtained from the mice.

The thickness of the material on the target backing is important for two reasons: (1) in a thick target, the protons lose a considerable amount of energy in traversing the sample and since the x-ray production cross section is a function of the proton energy, the x-ray yield is diminished; and (2) a thick target results in more self-absorption of x rays within the sample, again, resulting in fewer protons seen by the detector. These factors must be corrected or accounted for in the calibration of the system. However, if two samples have similar composition (i.e. bovine liver and mouse melanoma tumor) and if the targets are made uniformly (i.e. in the amount of material and thickness) then the proton slowing-down characteristics and

sample self-absorption should also be the same. The goal in target preparation was to make all targets, standards, and mice tissues the same.

At least nine targets of each standard and reference material were spiked and prepared as described above (and in Appendices G and H). Since the silver dopant added to all samples was the standard by which all other concentrations were compared, the calibration curve (Figure 36) is derived from the ratio of the net peak area for each element divided by its certified concentration value, to the net area of the silver peak divided by its concentration. Mathematically, the calibration factor equals:

$$\frac{\text{element net counts/ppm (NBS certified value)}}{\text{silver net counts/ppm (calculated from dopant added)}}$$

Note: Concentrations may be expressed either as ppm or, if known, μg per cm^2.

Figure 36 also shows data plotted from thin-film standards received from Gent, Belgium, which were discussed above. These standards were prepared especially for PIXE calibration and sensitivity determinations, so that there were no spectra-line interferences between elements.

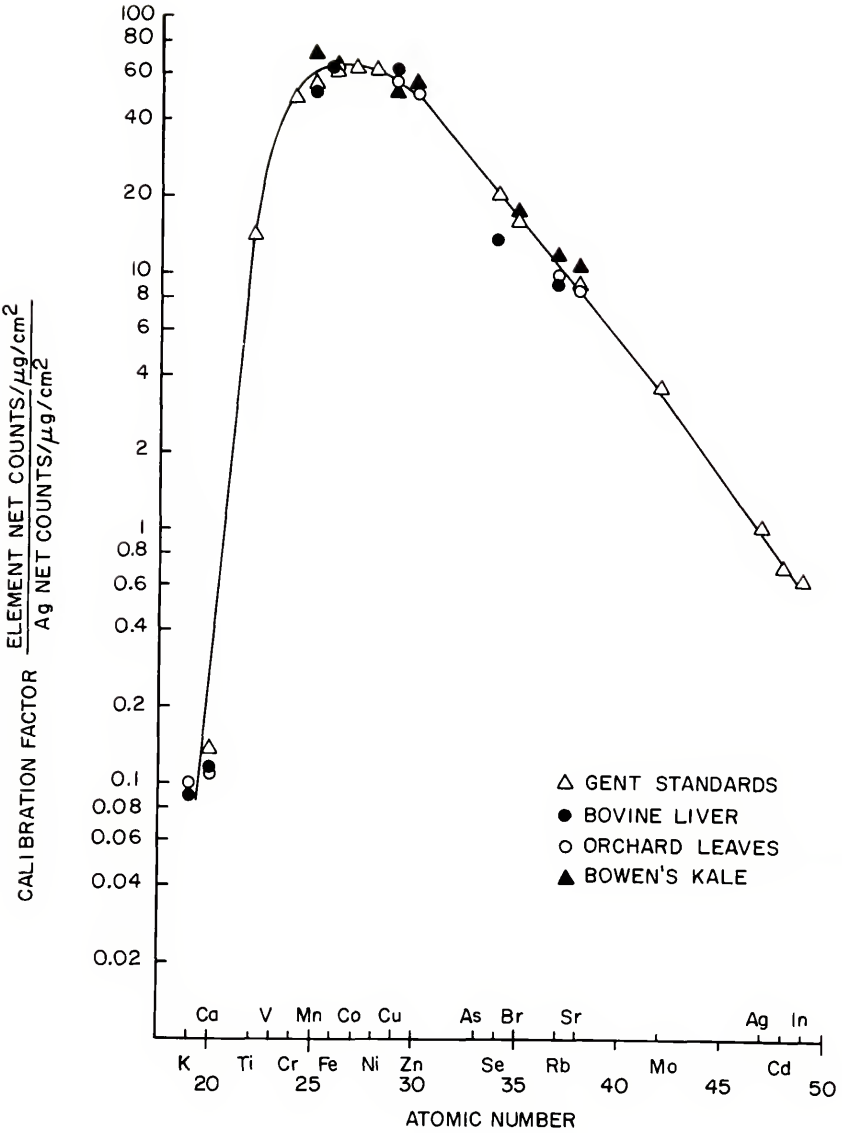
Quantitative Analysis

Once the calibration curve was established, the analysis of experimental samples was possible by the same standardization relationship which prevailed with the standards. Therefore, the ratios of the net counts in the sample spectrum for each element and the net counts for silver was determined. The concentration of silver in the sample was known, since it was added, so the elemental sample concentration was calculated from the relationship.

$$\text{element ppm} = \frac{\dfrac{\text{element net counts (sample)}}{\text{silver net counts (sample)}}}{\dfrac{\text{element net counts/ppm (standard)}}{\text{silver net counts/ppm (standard)}}} \times \text{silver ppm in sample}$$

FIGURE 36. CALIBRATION CURVE

The calibration curve was produced by spiking the "standard" reference materials, bovine liver, and orchard leaves, and the reference material Bowen's Kale powder, with silver nitrate solution. The silver acted as the internal-standard by which all other component elements were measured. The curve represents the ratio of the net integral counts for each element, divided by its certified concentration, to the net integral counts for silver, divided by its concentration in the sample, as a function of the atomic number of the constituent elements. The maximum, in the vincinity of maganese and iron imply that these elements can be detected in lower concentrations than elements of lower or higher atomic numbers. The "Gent Standards" are thin-film standards, especially prepared for PIXE analysis (described in the text).


CALIBRATION FACTOR ELEMENT NET COUNTS/μg/cm² / Ag NET COUNTS/μg/cm²
100
80
60
40
20
10
8
6
4
2
1
0.8
0.6
0.4
0.2
0.1
0.08
0.06
0.04
0.02
△ GENT STANDARDS
● BOVINE LIVER
○ ORCHARD LEAVES
▲ BOWEN'S KALE
Ca V Mn Co Cu As Br Sr Ag In
K Ti Cr Fe Ni Zn Se Rb Mo Cd
20 25 30 35 40 45 50
ATOMIC NUMBER

The denominator comes from the calibration curve, while the net integral counts in the peak of each element of interest was determined on the multichannel analzyer by establishing regions-of-interest bars on either side of the peak. The analyzer calculated the gross and net integral counts for any region of interest. The gross and net integrals were read, and recorded for ten regions of interest for approximately 270 spectra.

Corrections for Interfering Spectra Lines

A correction had to be applied to the integral count for some elements due to the interference (overlap) from neighboring elements. For example, the Kβ x rays from potassium (3.589 keV) and the Kα x rays from calcium (3.690 keV) are so close in energy that the detector could not resolve them individually, thus, they appeared as a single peak on the pulse-height spectrum. This meant that for samples in which both elements were present, (virtually every biological sample), the net calcium Kα peak had to be calculated from the overlapping peaks. The ratio of Kβ to Kα x rays is constant for each element, so by measuring the net integral for the Kα peak, the net area of the Kβ peak was calculated and subtracted from the overlapping peak area. This was the case in this example: there were no interfering lines in the potassium Kα peak; therefore, after its net integral was known, the area of the Kβ line could be calculated and subtracted from the calcium Kα peak. Even though the Kβ/Kα ratios have been published in the literature, they are a function of the system by which they are measured; therefore, it was not feasible to use published ratios. The type and thickness of absorber, and the window of the detector are factors which influence the ratios from one system to another. These ratios were determined for the analytical parameters used in this research by the "Gent standards" and are shown in Table 1.

TABLE 1

RATIOS OF Kβ TO Kα X-RAY YIELD FOR SELECTED ELEMENTS

Element	Kβ/Kα	Element	Kβ/Kα	Element	Kβ/Kα
Potassium	0.830	Cobalt	0.151	Rubidium	0.188
Calcium	0.616	Nickel	0.123	Strontium	0.165
Titanium	0.291	Copper	0.123	Molybdenum	0.200
Vanadium	0.260	Zinc	0.162	Silver	0.192
Chrominum	0.225	Arsenic	0.168	Cadmium	0.198
Manganese	0.238	Selenium	0.158	Indium	0.177
Iron	0.172	Bromine	0.122		

Corrections for Thick Targets

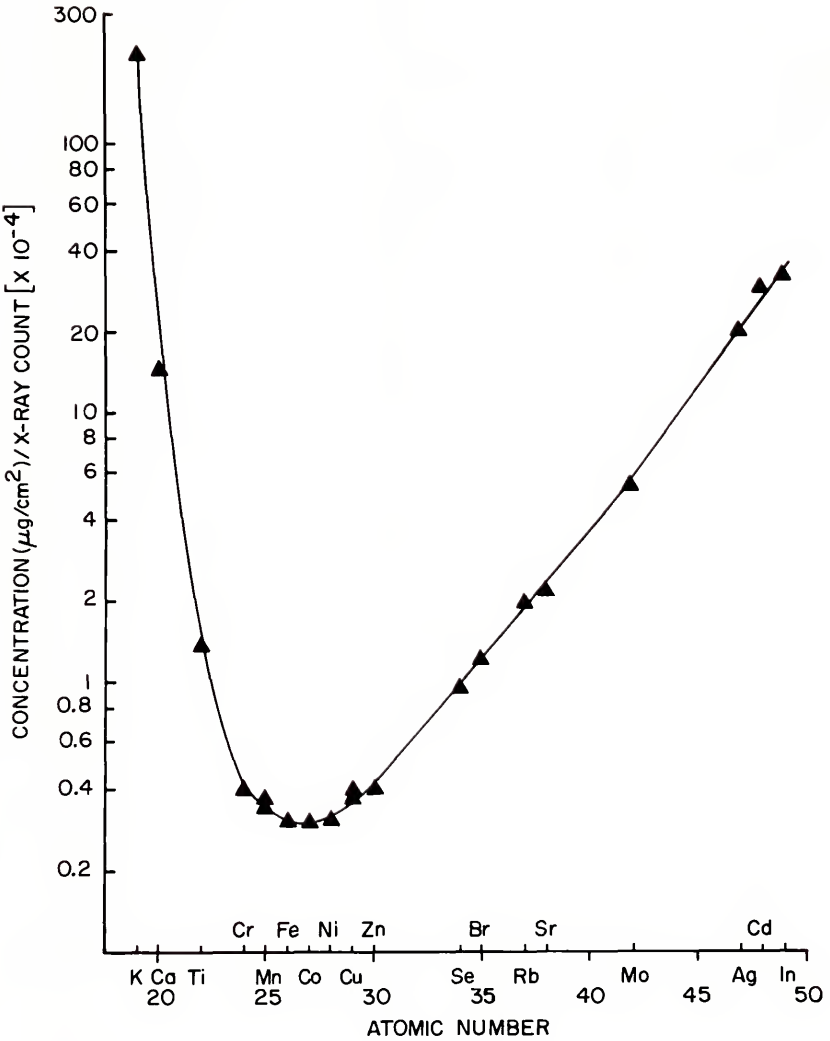
As mentioned previously, the loss of energy of the protons as they slow down in the target affects the ionization cross section, which results in a decline in x-ray production. In addition, a thick target absorbs a greater proportion of the protons before they reach the detector; therefore, theoretical calculations have been made to correct for these two phenomena in which the matrix was assumed to be Mylar. These correction factors (Van Rinsvelt and Maenhaut 1979) were included in the quantitative analysis calculations.

Sensitivity

One of the principle features of the PIXE method is its high sensitivity. The sensitivity of an analysis system is a measure of its ability to detect small concentrations of an element. It is sometimes called the lower limit of detection (LLD) or minimum detectable amount. The shape of the sensitivity curve for PIXE as shown in Figure 37, shows a minimum (maximum sensitivity) in a small range on the atomic number scale. The decrease in sensitivity for the lighter elements is due mainly to two factors: (1) the energies of

FIGURE 37. SENSITIVITY CURVE

This curve represents the lower limit of detection for the experimental parameters set in this investigation. Data were measured from the "Gent Standards" utilizing a proton energy of 2.5 MeV. The minimum in the curve, which represents the region of elements to which the system is most sensitive, can be shifted toward a particular element of interest by varying the energy of the bombarding particle, as discussed in the text.


300
100
80
60
40
20
10
8
6
4
2
1
0.8
0.6
0.4
0.2
CONCENTRATION (μg/cm²) / X-RAY COUNT [X 10⁻⁴]
Cr Fe Ni Zn Br Sr Cd
K Ca Ti Mn Co Cu Se Rb Mo Ag In
20 25 30 35 40 45 50
ATOMIC NUMBER

the Kα x rays are relatively small (1 to 2 KeV), thus more are absorbed within the target and the absorber; and (2) the fluorescence yield is small for the low Z elements and increases with atomic number.[1] The decreasing sensitivity for the high Z elements is due primarily to the fact that the x-ray production cross section decreases with increasing Z. An interesting fact to note about the sensitivity curve is that the maximum sensitivity can be shifted along the atomic number axis due to the change in cross sections with proton energy. This means that, within certain limits, it is possible to adjust the proton energy to achieve maximum sensitivity for a particular element (Johansson and Johansson 1976). If the proton energy has been increased, the maximum sensitivity would shift toward higher Z values. Data plotted in Figure 37 were derived from analyses of the thin-film standards from Gent, measured with a proton energy of 2.5 MeV.

Sources of Error

The errors considered in these analyses were classified by source and where absolute magnitudes were not calculable, estimates were given:

Statistical counting error. This error depends only on the total accumulated count and was calculated for each peak. Counting errors were generally below 3% for all elements analyzed except selenium, where low count rates caused this error to range from 7 to 15%.

Instrumental errors. These consisted of short-term and long-term variation, instability, and drift in instruments used in the analysis such as the detector, pulse processor, and multichannel analyzer. From the

[1] The fluorescence yield is the ratio of the number of x rays emitted, e.g., from K-shell vacancies, to the number of vacancies created in the K-shell. The competing reaction is the production of Auger electrons, in which an orbital electron is emitted from the atom instead of an x ray.

beginning to the completion of data acquisition, parameters such as energy resolution, counting rates, and centroid peak shifts were recorded and no significant changes were observed. This error contributed little to the overall error and was arbitrarily set at 3%.

Operational errors. These are errors resulting from nonreproducibility in setting instruments or the accelerator. No variations were observed which were attributable to operational errors; however, because they may have been present, the error was set at 3%.

Specimen errors. The errors associated with producing targets were considered the largest source because they impacted on the calibration curve as well as the experimental samples. The entire target-making process is comprised of a number of sources for error, for example: (2) weighing the samples, (b) error in the concentration of the spiking solution, (c) spiking the samples, and (d) the physical attributes of the sample itself, such as homogeniety, particle size, surface texture, and thickness. In addition to these factors, the reference standards from NBS had errors which ranged from 4 to 10% for the certified values of elemental concentrations. With consideration given to all these factors, the specimen error was set at 15%.

The overall error was calculated to be approximately 25%, which is generally considered to be appropriate for this type of analysis (Van Rinsvelt 1980).

Example Quantitative Calculation

To aid in understanding the steps involved in arriving at a concentration from the net integral counts on a spectrum, an example will be shown taken from the actual data:

Calculate the quantity of zinc in target number two of sample number seven, given the following data:

zinc Kα net integral counts = 46231,

silver Kα net integral counts = 32907,

silver concentration in the target - 2695 ppm, and

"thick" target correction factor = 0.895

Due to the interference of the copper Kβ line, the zinc Kα net counts must be corrected as follows:

copper Kα net integral counts = 3315;

From Table 1, the Kβ/Kα ratio for copper = 0.123.

Therefore, the adjusted zinc counts = 46231 - 0.123 (3315) = 45823.

The calibration factor for zinc = 50, therefore,

$$\text{ppm zinc} = \frac{45823/32907}{50} \times 2695 \text{ ppm} \times 0.895 = 67 \text{ ppm} +/- 17 \text{ (dry weight)}.$$

A minimum of three targets was analyzed for each sample and the mean was taken as the representative concentration for a particular element.

CHAPTER IV

RESULTS AND DISCUSSIONS

Eight elements--potassium, calcium, iron, copper, zinc, selenium, bromine, and rubidium--were found consistently in all specimens except for rubidium which was below the lower limit of detection in serum samples. Occasionally other elements, such as nickel would appear in the spectra, but their presence was so irregular and the quantity so small that it was not feasible to quantitate them.

Primary Tumor Versus Metastatic Tumor

The elemental concentration profiles for the B-16 primary and metastatic tumor tissues are summarized in Table 2. The variations in the number of observations for the subcutaneous tumor were due to rejecting outlying datum whenever the value exceeded three standard deviations. The distribution of elemental concentrations was assumed to be approximately normally distributed which implies that about 99% of the data falls within three standard deviations of the mean. In actual fact, the observations rejected (one each for Ca, Se, and Rb and two for As) were more than four standard deviations from the mean. It should be kept in mind that the total number of targets analyzed was approximately three times the "N" shown in the table. For example, Table 2 shows there were 16 metastatic tumor samples; however, the mean elemental concentrations were determined by analyzing from three to five targets prepared from each sample.

Table 2 shows that the largest sampling variation occurred for iron, which was not surprising since it is a major component of blood and both tissue types are highly vascularized and ladened with blood because of

TABLE 2

SUMMARY OF ELEMENTAL PROFILES
B-16 MELANOMA PRIMARY AND METASTATIC TUMOR

Element	N	Mean(PPM)	STD DEV	MIN	MAX	CV%*
			SUBCUTANEOUS TUMOR			
K	12	14619	2818	11049	20016	19.3
Ca	11	798	173	562	1215	21.6
Fe	12	272	117	108	460	43.0
Cu	12	6	1	5	9	20.6
Zn	10	68	6	62	81	8.3
Se	11	1.51	0.25	1.18	1.96	16.6
Br	12	21	2	19	25	9.0
Rb	11	14.1	2.9	10.3	18.5	20.6
			METASTATIC TUMOR			
K	16	15378	3728	9705	22339	24.2
Ca	16	251	120	118	491	47.9
Fe	16	276	129	117	576	46.6
Cu	16	4	1	3	6	18.4
Zn	16	76	11	59	103	14.2
Se	16	1.63	0.31	1.06	2.21	19.4
Br	16	4	2	2	7	35.2
Rb	16	16.2	4.0	9.3	23.4	24.6

* coefficient of variation (%)

the demands of the rapidly dividing cancer cells. Although all specimens were rinsed thoroughly with water, the internal collection of blood remained in the samples and the degree of cleansing of the blood was highly variable among samples. In metastatic tumor, a large fluctuation is shown for calcium concentrations, probably due to the cleansing operation since it,too, is abundant in blood.

When comparing mean elemental concentrations between the two tissue types, some rather large differences appeared such as for copper; therefore, the data were analyzed by the Student's t-test. The summary of the statistical tests is shown in Table 3. A significance level of 0.01 was chosen since there was the likelihood of a test inadvertantly showing a difference at the 0.05 level due to calculating so many individual t-tests. By decreasing the significance level, more confidence could be placed in the results.

Calcium, copper, and bromine were all found in significantly higher levels in primary tumor tissue than in the lung metastases. However, metastatic tumor showed higher concentrations of zinc and rubidium than primary tumor, but at lower levels of significance. Since calcium is the most abundant element in the body (in man, at least), it is not surprising to find it, but the level in the primary tumor is over twice that in either metastasis or control lung tissues.

Copper is an essential element in the formation of hemoglobin and has been reported at elevated levels in the serum of certain cancer patients. Apparently the body uses the copper as some defense against the invading cancer. Increased dietary intake of copper has been shown to inhibit tumor induction in rats (Underwood 1977).

Although bromine is not considered an essential element, it is one of the most abundant in the biosphere. It is apparently being used by the

TABLE 3

SUMMARY OF t-TESTS

(H_o: $\mu_1 = \mu_2$)

	Primary Tumor vs. Metastatic Tumor			Primary Tumor vs. Control Lungs			Metastatic Tumor vs. Control Lungs		
	t	t-crit	Comment	t	t-crit	Comment	t	t-crit	Comment
Potassium	0.588	2.479	*	1.240	2.681	*	1.225	2.583	*
Calcium	9.733	2.485	Reject H_o	3.930	2.718	Reject H_o	0.537	2.583	*
Iron	0.085	2.479	*	0.481	2.681	*	0.399	2.583	*
Copper	4.923	2.479	Reject H_o	5.900	2.681	Reject H_o	8.780	2.583	Reject H_o
Zinc	2.110	2.492	*	3.572	2.764	Reject H_o	0.969	2.583	*
Selenium	1.050	2.485	*	0.163	2.718	*	0.653	2.583	*
Bromine	19.858	2.479	Reject H_o	3.959	2.681	Reject H_o	9.933	2.583	Reject H_o
Rubidium	1.492	2.485	*	0.908	2.718	*	0.065	2.583	*

* Insufficient evidence to reject the null hypothesis.

body in some important role regarding the cancerous growth since it has been reported (Cole and Patrick 1958) to not accumulate in any particular area of the body, yet the concentration in the primary tumor was over fives times greater than in the metastasis. It is also interesting to note that the levels of bromine in metastases are significantly less than normal tissue.

Serum and Lung

Although quantitative analyses were done on serum samples from experimental and control mice, the number of samples is so small that little more than the presence and relative magnitudes of the elements can be taken with assurance. The same is true for control and experimental lung tissue. The first objective was to characterize the elemental content of the primary and disseminated tumor, and analyses of the relatively few lung samples were done to attempt to corroborate the INAA technique results. The sera and lung data are tabulated in Tables 4 and 5, respectively. Since the experimental sera came from different groups of mice, once which was injected subcutaneously with the B-16(F1) cell line and the other group which received intravenous injections of B-16(F10) cells, it was not appropriate to combine the data; therefore, each data set is presented in Table 4. Student's t-test were conducted to measure differences in the concentration means for the two experimental serum groups. No significant differences were seen at the $p = 0.05$ level; however, due to small number of degrees of freedom (3), the test is weak. Similar results came from t-tests between experimental and control serum groups. More data are needed to test for changes in elemental serum levels with the state of health and extent of the disease. It is interesting to view the data qualitatively, for although the difference in serum copper levels is not "statistically different" the trend is for both

TABLE 4

SUMMARY OF ELEMENTAL PROFILES CONTROL AND EXPERIMENTAL SERA

ELEMENT	N	MEAN(PPM)	STD DEV	MIN	MAX	CV%*
			CONTROL SERUM			
K	5	2314	180	2045	2499	7.8
Ca	5	1210	77	1154	1342	6.4
Fe	5	27	8	21	41	31.4
Cu	5	7	1	6	9	17.5
Zn	5	11	1	10	13	11.1
Se	5	3.66	0.26	3.25	3.89	7.0
Br	5	87	3	84	90	3.3
Rb	5	<LLD	NA	NA	NA	NA
			EXPERIMENTAL-2 SERUM			
K	2	3010	712	2507	3514	23.7
Ca	2	1526	192	1390	1661	12.6
Fe	2	34	2	33	36	6.1
Cu	2	8	1	7	8	9.4
Zn	2	10	1	10	11	6.7
Se	2	3.53	0.72	3.02	4.04	20.4
Br	2	101	10	94	108	10.1
Rb	2	<LLD	NA	NA	NA	NA
			EXPERIMENTAL-3 SERUM			
K	3	2592	494	2113	3099	19.0
Ca	3	1205	16	1187	1215	1.3
Fe	3	21	2	20	24	10.8
Cu	3	9	1	8	10	11.1
Zn	3	9	1	9	10	6.2
Se	3	4.22	0.16	4.09	4.40	3.8
Br	3	104	19	85.2	124	18.5
Rb	3	<LLD	NA	NA	NA	NA

* Coefficient of variation (%)

TABLE 5

SUMMARY OF ELEMENTAL PROFILES
CONTROL AND EXPERIMENTAL LUNGS

Element	N	Mean(PPM)	STD DEV	MIN	MAX	CV%*
			CONTROL LUNGS			
K	2	12060	514	11697	12424	4.3
Ca	2	298	45	267	330	14.9
Fe	2	314	47	280	347	15.1
Cu	2	17	7	12	22	41.6
Zn	2	84	5	80	87	5.9
Se	2	1.48	0.7	1.43	1.53	4.8
Br	2	16	0.4	15.4	16	2.7
Rb	2	16.0	1.3	15.1	17.0	8.4
			EXPERIMENTAL LUNGS			
K	3	9652	259	9471	9949	2.7
Ca	3	225	22	203	246	9.6
Fe	3	236	29	206	263	12.1
Cu	3	11	1	10	12	9.1
Zn	3	69	3	67	73	4.7
Se	3	1.32	0.19	1.14	1.52	14.4
Br	3	14	1.15	13	15	8.4
Rb	3	13.3	1.0	12.3	14.2	7.2

* Coefficient of variation (%)

experimental groups to have more copper in the serum than do the controls. Yet, if there is more copper circulating in the blood, and the same blood eventually passes through the primary and the metastatic tumor, why is the primary tumor concentration significantly higher than the metastases?

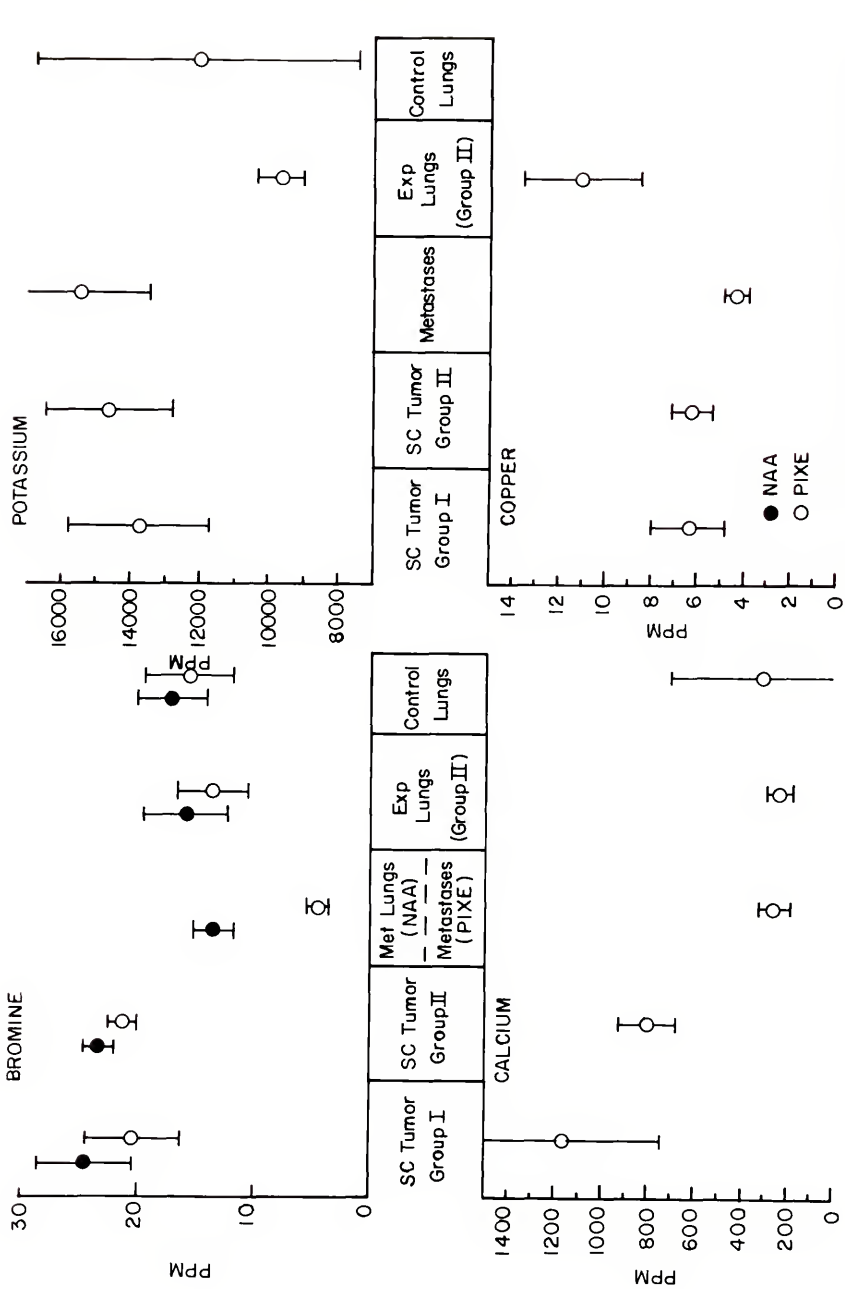
The bromine level in subcutaneous tumor is approximately five times the level in pulmonary metastases. These data are shown in Figure 38, where a comparison is also made with the INAA technique for bromine, one of five elements the two analytical techniques had in common. The bars represent the 95% confidence interval based on a t-distribution. The large intervals around the mean-lung concentrations are due to the fact that there were only two control and three experimental samples analyzed by PIXE. Those values were included in the figure only for informational purposes and should not be considered statistically representative of the true elemental concentrations.

Complimentarity of PIXE and INAA

Results from split sample analyzed by the neutron activiation technique agreed and supported the PIXE results extremely well. Five elements overlapped and were analyzed by both techniques, namely: bromine, zinc, iron, selenium, and rubidium. A summary of the combined data is shown in Figures 38 through 40. Highly elevated levels of bromine in primary tumor samples was confirmed by each technique. There is a lower level of bromine in both the individual metastases and metastatic lung than in control lungs. It must be pointed out again that for PIXE analysis, there were only two control lung samples. Although the PIXE data failed to corroborate the INAA data for selenium analysis, this metal was found in higher concentrations in both primary tumor and metastatic lung tissue than in control which is in agreement with other cancer investigations which

FIGURE 38. PIXE/INAA COMPARISON OF RESULTS-1 (Bromine, Calcium, Potassium, Copper)

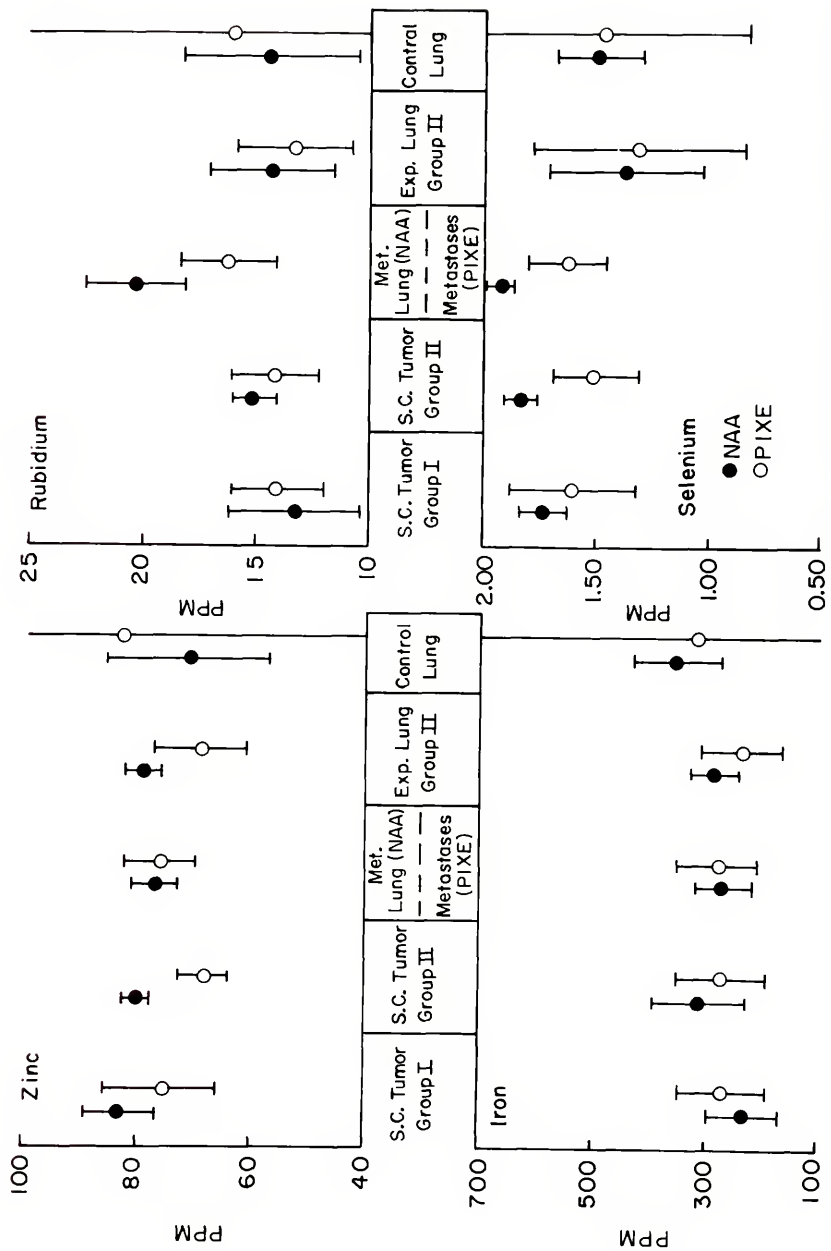
Shown are the mean values for the elemental concentrations measured by PIXE analyses (PIXE and INAA values are compared for bromine). The error bars represent the 95% confidence interval based on a t-distribution. The large range for the control lung samples is due to the fact that only two samples were analyzed by the PIXE technique. Although depicted here for purposes of comparison, the Group I subcutaneous tumor and Group II experimental lung results were not used in subsequent t-tests because the treatments introduced experimental differences which precluded comparison. Note that there are significant differences in the levels of calcium and copper when comparing tumor with metastases.



Note: INAA data furnished by Swanson (1980).

FIGURE 39. PIXE/INAA-2 COMPARISON OF RESULTS (Zinc, Iron, Rubidium, Selenium)

Shown is a confidence interval comparison for elements detected by both the PIXE and INAA analytical techniques (legend in lower right quadrant). All error ranges represent a confidence level of 95% based on a t-distribution. The large error bar range for PIXE control lung data is due to the small number of samples (2). Although depicted here for purposes of comparison, the Group I subcutaneous tumor and Group II experimental lung results were not used in subsequent t-tests because the treatments introduced experimental differences which precluded comparison. With the exception of zinc and selenium in Group II subcutaneous tumor, all other comparison results (for the same sample material) were favorable.



Note: INAA data furnished by Swanson (1980).

FIGURE 40. COMBINED ANALYSES OF INAA AND PIXE

The "Student's t" test statistic was used to determine whether differences existed between sample populations. Because many individual t-tests were made there was an increased probability that a difference would be considered significant at some confidence level when, in fact, it was not. For this reason, only highly significant ($p < 0.005$) were considered conclusive. Other results considered marginally significant are shown along with their associated p-value.

Of particular note are the following:

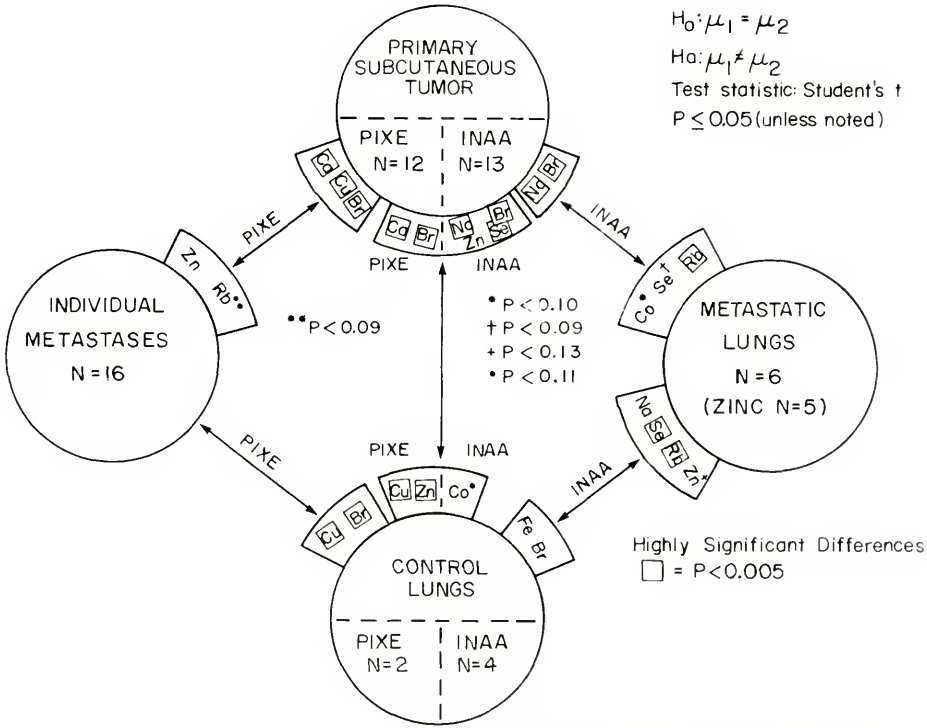
1. There is more selenium in cancer tissue (both primary tumor and metastatic) than in normal control tissue. With regard to the primary tumor, this is in agreement with current findings by other investigators. The same finding with metastasis had been expected but not previously documented. Selenium has been considered a "cancer inhibiting" agent in recent years.

2. Primary tumor and control tissue do not differ in rubidium concentration while metastatic lung tissue and (to a lesser extent) individual metastases have significantly higher concentrations. Rubidium is not considered an essential element, but its presence in animals is, for some unknown reason, greater than would be expected from environmental considerations alone, indicating some physiological regulating mechanism. The fact that, in most cases, rubidium concentrations varied in opposition to calcium, bromine, and sodium indicates that the rubidium is present in some non-ionic form.

3. Increased cobalt was found in both cancerous and control lungs relative to the primary tumor. This indicates that cobalt is probably a component of lung tissues.

4. Lower concentrations of copper were found in individual metastases than in the primary tumor with less in the primary tumor than in control lungs.

COMBINED ANALYSES
INAA AND PIXE



Note: INAA data furnished by Swanson (1980).

showed that selenium is taken up in fast-growing neoplasms and apparently has a tumoricidal or protective effect.

Differences are seen in the rubidium level between primary tumor and metastasis. INAA results confirm the observation that metastasis and metastatic lung have increased uptake of rubidium over the other samples. The only disparity between the two techniques was in the distribution of zinc between primary tumor and control lungs. PIXE analysis found that control lungs have more zinc than the primary tumor and the INAA found just the opposite. The concentrations of zinc found by the two techniques agreed quite closely, but the fact that the only two samples of control lung were analyzed by PIXE probably accounts for the disparity. The statistics are not sufficient to say that the difference found by PIXE is significant.

CHAPTER V

SUMMARY AND CONCLUSIONS

The primary objective of this research was to determine the trace element profile of B-16 murine melanoma, and specifically to investigate whether differences in elemental composition exist between a primary tumor and metastasis. Twelve primary subcutaneous tumor specimens, 16 individual metastases, five experimental and five control serums, and two control lung samples were analyzed by the particle-induced x-ray emission (PIXE) technique.

The first step in achieving this objective was to inject viable cancer cells into three groups of mice according to the following scheme:

(1) Group I mice were given a subcutaneous injection of B-16(F1) melanoma cells into the right flank. Approximately two weeks later, these mice were given an intravenous injection of more cancer cells.

(2) Group II mice received only a single, subcutaneous injection of B-16 (F1) cells.

(3) Group III mice were administered an intravenous injection of tumor-producing B-16(F10) cells via a lateral tail vein.

A large primary tumor developed at the site of the subcutaneous injection, while the intravenous inoculum, being carried in the blood stream, established tumor colonies in the lungs. These mouse tumors became the population from which samples were drawn and analyzed in this research.

In all, 8 elements (potassium, calcium, iron, copper, zinc, selenium, bromine, and rubidium) were consistently found in all samples except for

rubidium, for which the concentration in serum fell below the lower limit of detection.

A statistical test (Student's-t) was calculated for each element-pair to determine if differences in mean concentrations were significant (at $p = 0.01$) between primary tumor and metastasis. Three elements showed highly significant differences, calcium, copper, and bromine. Due to so few observations for the serum and lung samples, the tests for differences in concentrations were too "weak" to be of use and only qualitative "trends" could be derived.

The subcutaneous tumor samples were ground into a fine powder, homogenized, and split so that in addition to PIXE analysis, a portion could be analyzed by the complementary technique of instrumental neutron activation analysis (INAA). Each technique independently analyzed eight elements. In addition, five elements were analyzed in common by both methods. Each technique complemented the other for all elements except zinc. The mean values for zinc were very close between the methods, but the slight trend which did occur showed more zinc in control lungs than primary tumor for PIXE analysis and just the opposite for INAA.

Significant progress which has come out of this research can be summarized as follows:

Techniques were developed,

(1) to handle viable B-16 melanoma cells--this included the culturing, harvesting, cryopreserving, and inoculum preparation of the cells,

(2) to work on the animals--including injecting, sacrificing, performing surgery, and collecting specimens,

(3) to optimize the analytical technique--such as sample preparation, target preparation, operation of the Van de Graaff accelerator and detector/analyzer system, and data acquisition and analysis.

This was a "pilot" project to see whether this type of research could be accomplished by bringing together the resources of three major disciplines within the University. The results are highly encouraging in two ways: (1) this effort proved that the mechanics for this type of research are feasible, and, more importantly, (2) the research showed that there are significant differences in the trace element concentrations between primary B-16 melanoma and its metastases. There is sufficient evidence to indicate that the role of trace elements in cancer metastasis should be continued.

APPENDIX A

NON-STANDARD LABORATORY
MATERIALS AND EQUIPMENT

1.	Item	Anesthetic
	Pur.	Anesthetize mice
	Model	Methoxyflurane
	Mfgr.	Abbot Laboratories, North Chicago, IL
2.	Item	Balance
	Pur.	Weigh samples/standards
	Model/size	Type B6
	Mfgr.	Mettler Instrument Corp., Hightown, NJ
3.	Item	Bovine Liver Standard
	Purpose	Calibration standard
	Model/size	SRM 1557
	Mfgr.	National Bureau of Standards, Washington, DC
4.	Item	Bowen's Kale Powder
	Pur.	Calibration reference
	Mfgr.	H.J.M. Bowen, Chemistry Dept., University of Reading, Berkshire, England
5.	Item	Centrifuge, refrigerated
	Pur.	Spin B-16 melanoma cells
	Model/size	RC-3
	Mfgr.	Sorvall
6.	Item	Centrifuge tubes
	Pur.	Spinning B-16 melanoma cells; preparing inoculum
	Model/size	Polypropylene, 50 ml
	Mfgr.	Rochester Scientific Co., Inc., Rochester, NY

7.	Item	Cryovials
	Pur.	Deep freeze storage of B-16 melanoma cells; sample containers
	Model/size	Provial
	Mfgr./Supp.	Fisher Scientific Co.
	Comment	High density, polyethylene. 2 ml volume.
8.	Item	Dextrose
	Pur.	Washing tumor and lung samples
	Model	5% in water
	Mfgr.	Travenol Laboratories, Inc., Deerfield, IL
9.	Item	Ethylenediamine tetracetic acid (EDTA)
	Pur.	Remove B-16 cells from tissue culture flasks
	Model	Sigma Grade
	Mfgr.	Sigma Chemical Co., St. Louis, MO
10.	Item	Filter
	Pur.	Sterilize tissue culture media
	Model/size	Millex 0.6, 0.45, 0.22 μm
	Mfgr.	Millipore Corporation, Bedford, MA
11.	Item	Incubator
	Pur.	Culture B-16 melanoma cells
	Mfgr.	Forma Scientific
	Comment	7% CO_2, humidified, 37°C
12.	Item	Ingredients for complete minimum essential media (Appendix B)
	Pur.	Tissue culture medium for B-16 melanoma cells
	Mfgr.	Grand Island Biological Company, Grand Island, NY 14072
13.	Item	Mice
	Pur.	Syngenic host for B-16 melanoma
	Model	C57BL/6j, black mice
	Mfgr.	Jackson Labs, Bar Harbor, ME
	Comment	Male, 6-8 weeks old
14.	Item	Multichannel analyzer
	Pur.	Acquiring x-ray spectra
	Model	TN-1710
	Mfgr.	Tracor Northern

15. Item — Orchard Leaves Standard
    Pur. — Calibration standard
    Model/size — SRM 1551
    Mfgr. — National Bureau of Standards, Washington, DC

16. Item — Planer Programed Freezer
    Pur. — Freeze B-16 melanoma cells for cryopreservation
    Model — R20154

17. Item — Silver Nitrate crystals
    Pur. — Internal-standard dopant
    Model — AG75
    Mfgr. — Spex Industries, Metuchen, NJ

18. Item — Syringe, glass
    Pur. — Inoculating mice
    Model/size — 0.5 ml, sterile, disposable
    Mfgr. — Becton, Dickinson and Co., Rutherford, NJ

19. Item — Tissue culture flasks
    Pur. — Culture B-16 melanoma cells
    Model/size — 75 cm^2 -250 ml
    Mfgr. — COSTAR, Cambridge, MA

APPENDIX B

INGREDIENTS OF TISSUE CULTURE MEDIUM
FOR
B-16 MELANOMA TUMOR CELLS

Materials:

Minimum essential medium with Hanks' salts	850 ml
Fetal bovine serum	100 ml
Non-essential amino acids	10 ml
Sodium pyruvate	10 ml
Penicillin-streptomycin	5 ml
Minimum essential medium vitamin solution	15 ml
L-glutamine	10 ml
$NaHCO_3$ 7.5% solution	30 ml

Sterile water (1 liter)

Filters (0.45 and 0.22 µm)

Containers in which to store the complete medium

pH testing paper

1. Warm all ingredients to room temperature.

2. Mix ingredients in the order listed.

3. Do all mixing in a sterile hood.

4. Test the pH of the final mixture and adjust if necessary with $NaHCO_3$ to obtain pH in the range of 7.2 to 7.5.

5. Filter the medium through a 0.45 µm filter, then through a 0.22 µm filter to remove any contamination

6. Store the complete minimum essential medium (CMEM) in the refrigerator at $12^{o}C$.

NOTE: All ingredients were from Grand Island Biological Company, Grand Island, NY. See Appendix A for a complete listing of manufacturers and suppliers of materials and equipment.

NOTE: L-GLUTAMINE MUST BE REPLENISHED AT TWO WEEK INTERVALS

APPENDIX C

PROCEDURE FOR SPLITTING AND RECULTURING B-16 MELANOMA CELLS

Materials:

Complete minimum essential medium (CMEM - see Appendix B)

2 mM ethylenediamine tetraacetic acid (EDTA)

Tissue culture flasks (250 ml*)

Syringe (60 ml)*

Filter (0.22 μm)

Centrifuge tubes (50 ml)*

Pipettes (5 and 10 ml)*

*(sterile, plastic, disposable)

1. Warm the EDTA and CMEM to room temperature. (They are stored in a refrigerator at 10°C.

2. Filter the EDTA and CMEM through the 0.22 μm filter to insure sterility.

3. Tighten the caps on the tissue culture flasks before removing them from the incubator.

4. Gently swirl the medium around in the flask to loosen and suspend the dead cells. Viable cells are firmly attached to the bottom.

5. Pour off the supernatant cell suspension.

6. Add 2 ml of EDTA to the flask, shake gently for 15 to 30 seconds, then pour off.

7. Add another 0.5 ml of EDTA. Tap the flask firmly to loosen the cells from the bottom of the flask. Make sure the cells are free.

8. Add 2 ml of fresh CMEM to the flask. Mix gently, but thoroughly with a pipette to break up any clumps of cells.

9. Add another 8 ml of CMEM to make a total of 10 ml. Mix thoroughly.

10. Divide the cell suspension into the required number of flasks, then bring each flask to a 10 ml volume with CMEM.

11. Shake gently to distribute cells uniformly over the bottom surface of the flask, making sure the entire bottom is covered.

12. Replace and tighten the caps on the flasks while still in the hood.

13. Transfer the flasks to the 37°C incubator with 7% CO_2 atmosphere, then loosen the caps on the flasks in the incubator. The CO_2 atmosphere is necessary to maintain the proper pH of the medium.

APPENDIX D

PROCEDURE FOR CRYOPRESERVATION
OF
B-16 MELANOMA CELLS

Materials:

Complete minimum essential medium (CMEM - see Appendix B)

Dimethyl sulfoxide (DMSO)

Ethylenediamine tetraacetic acid (EDTA)

Centrifuge tubes (50 ml)*

Syringe (60 ml)*

Filter (0.22 µm)

Pipettes (1, 5 and 10 ml)*

Cryovials (2 ml)

Ice

*(sterile, plastic, disposable)

1. Warm the EDTA and CMEM to room temperature.

2. Label all centrifuge tubes and cryovials.

3. Filter the EDTA and CMEM through the 0.22 µm filter with a 60 ml syringe into appropriately labelled centrifuge tubes.

4. Tighten the caps on the tissue culture flasks before removing them from the incubator.

5. Loosen the B-16 melanoma cells from the flask as in Appendix C.

6. After the cells are loosened, add 1 ml of CMEM to the flask to dilute the EDTA on the cells and combine all the cells into a single flask.

7. Pipette the cells into a labelled centrifuge tube.

8. Spin them in a refrigerated centrifuge ($12^{o}C$) at 500 rpm for 10 minutes.

9. Combine 16 ml of CMEM and 4 ml of DMSO in a centrifuge tube.

10. Following the spin, use a vacuum aspirator to siphon off the supernatant.

11. Resuspend the cells in the same centrifuge tube with 5 to 10 ml of CMEM. Use the force provided by a manual pipettor to separate any clumps of cells into individual cells.

12. Respin the cells in the centrifuge as above.

13. Again, siphon off the supernatant.

14. Resuspend the cells in 5 ml of CMEM. Break up the clumps of cells as before.

15. Place the centrifuge tube containing the cell suspension into a container of ice.

16. Take a drop of the suspended cells and assay them with a hemocytometer.

17. Adjust the cell suspension to approximately 1×10^6 cells per ml with the CMEM/DMSO solution.

18. Pipette 1 ml of cells per cryovial. Keep the cells on ice until placed in the freezer.

19. Transfer the vials to a Planer Programmed Freezer. After a temperature of $-40^{\circ}C$ is reached, manually lower the temperature to approximately $-120^{\circ}C$.

20. Transfer the frozen vials into storage racks and place them in a liquid nitrogen storage tank for preservation in the liquid nitrogen vapor phase.

APPENDIX E

PROCEDURE FOR THAWING AND RECULTURING
OF
B-16 MELANOMA CELLS

Materials:

Complete minimium essential medium (CMEM - see Appendix B)

Tissue culture flasks (250 ml)*

Syringe (60 ml)*

Filter (0.22 µm)

Centrifuge tubes (50 ml)*

Pipettes (5 and 10 ml)*

1. Warm CMEM to room temperature.

2. Label all centrifuge tubes and flasks.

3. Filter the CMEM through the 0.22 µm filter with a 60 ml syringe into an appropriately labelled centrifuge tube.

4. Prepare a 37°C water bath.

5. Remove frozen B-16 melanoma cells from the liquid nitrogen cryofreezer and thaw cells for no more than 90 seconds in the water bath.

6. Take the vials into the sterile hood before opening them.

7. Add 1 ml of CMEM to each vial of cells. Add medium "dropwise", not all at once. Gently shake the vials while adding the medium.

8. Using a manual pipettor and a 5 ml pipette, draw the cells up and down in each vial to break up any clumps.

9. Tranfer (pipette) the cells from each vial into the labelled centrifuge tubes; one centrifuge tube per vial.

10. At a rate of 1 ml per minute, add 8 ml of CMEM to each centrifuge tube and shake gently while adding the medium.

11. Gently mix the cells in the centrifuge tube with a clean pipette.

12. Pipette the cells into labelled tissue culture flasks. Make sure the medium covers the bottom completely.

13. Replace and tighten the caps of the flasks before removing them from the hood.

14. Place the flasks in a $37^{\circ}C$ incubator with a 7% CO_2 atmosphere.

15. Loosen the caps on the flasks.

APPENDIX F

PREPARATION OF B-16 TUMOR CELLS FOR INJECTION

Materials:

Complete minimum essential medium (CMEM - see Appendix B)

2 mM ethylenediamine tetraacetic acid (EDTA)

Hanks' balanced salt solution (HBSS)

Centrifuge tubes (50 ml)*

Pipettes (5 and 10 ml)*

Syringe (60 ml)*

Filter (0.22 µm)

Ice

Vortex vibrator

*(sterile, plastic, disposable)

1. Warm the CMEM, EDTA and HBSS to room temperature.

2. Filter the CMEM, EDTA and HBSS through the 0.22 µm filter with a 60 ml syringe into properly labelled centrifuge tubes.

3. Follow the steps in Appendix C for loosening the cells from the culture flasks.

4. Transfer the cells from the flasks into one centrifuge tube. The contents of several flasks may be combined.

5. Spin the cells in a refrigerated centrifuge ($12^{o}C$) at 500 rpm for 10 minutes.

6. Siphon off the supernatant and resuspend the pellet of cells in 5 ml of HBSS. Gently break up the pellet and clumps of cells with a manual pipettor.

7. Recentrifuge the cells at 500 rpm for 10 minutes.

8. Decant the supernatant and resuspend the cells in 5 ml of HBSS. Again, use the manual pipettor to break the clumps of cells apart.

9. Place the tube of cells in a container of ice. Thoroughly vibrate the cells at 5 to 10 minute intervals to prevent clumping.

10. Assay the total number of cells per ml with a hemocytometer.

11. Assay the cells for viability using the trypan blue exclusion test.

12. Dilute the cell suspension with HBSS to a concentration of approximately 1 x 10^5 viable cells per ml.

13. The cell suspension is ready for injections. The cells must remain on ice and be agitated frequently until drawn into the syringe for injection.

14. All steps must be done without delay because the cell viability is decreased in HBSS. All injections should be completed as soon as possible.

APPENDIX G

PROCEDURE FOR PREPARING TISSUE SAMPLES

Materials and equipment:

Polyethylene vials (2 ml)

Polyethylene centrifuge tubes (50 ml)

Filter paper

Dewar of liquid nitrogen

Lyophilizer (freeze-dryer)

Quartz rod (6 mm o.d.)

Balance

Micropipets (10, 20, and 50 µl)

Double-distilled deionized water

Oven (up to 80°C)

Spiking solution (silver nitrate - approximately 3 µg silver per µl)

Dextrose solution (sterile, 5%)

1. Rinse the fresh tissue with 5% dextrose immediately after excision.

2. Place the tissue in a centrifuge tube and cover with 5% dextrose solution during temporary storage.

3. After all specimens have been collected, rinse each one thoroughly in double-distilled deionized water to remove any blood, hair, or loose tissue.

4. Transfer samples into clean, labelled centrifuge tubes and cover with filter paper (secure it with a rubber band).

5. Immerse the tubes in the dewar of liquid nitrogen until the samples are frozen.

6. Place the samples in the lyophilizer for 24 to 48 hours.

7. Set the tubes in a styrofoam holder filled with liquid nitrogen to keep the samples frozen. Use the quartz rod to pulverize the tissue by the brittle fracture technique.

8. Return the samples to the lyophilizer for 24 hours.

9. Label and preweigh the 2 ml vials (one for each sample).

10. Transfer the powdered samples into the vials and cap tightly (work quickly to minimize absorption of moisture from the air).

11. Weigh the vials to determine the amount of sample added.

12. "Wet" the sample with double-distilled deionized water at a rate of 2 to 3 µl of water per mg of sample:

    i.e. if the sample = 70 mg ==> add 140 to 210 µl of water (the exact amount is not critical but should be within this range).

    NOTE: Do not add all the water in one shot - but, for example, 50 µl at a time. Let the water soak in before adding the next aliquot.

13. Leave the cap off and place the samples in the oven at 35 ^{o}C for 2 hours to dry the material slightly.

14. Spike the samples with the silver nitrate stock solution to approximately 3000 ppm:

    i.e., if the stock solution contains 3 g silver/liter = 300 µg silver/µl and the sample (dry weight) is 70 mg, then add 70 µl of solution.

15. Place samples in a 60^{o}C oven until the solution is "soaked-in".

16. Increase the oven temperature to 80^{o}C for 48 hours.

17. Replace the caps and store until ready for target preparation.

APPENDIX H

PROCEDURE FOR PREPARING SAMPLE TARGETS

NOTE: Do all work in the filtered-air hood.

Materials and equipment:

Polyethylene target rings (2.0 cm i.d., 2.5 cm o.d.)

Aluminized Mylar backing material

Spray adhesive (3M Company, Photo Mount)

Alcohol

Cleaning tissue

Petri dishes

Scalpel

Glass plate (approximately 15 cm in diameter)

Forceps

1% Formvar solution (Polysciences, Inc., Rydal, PA)

Micropipetter (10 µl with disposable plastic tips)

Double-distilled dionized water

Aluminum plate (for picking up Formvar film)

Rubber stopper (number five)

Agate mortar and pestle

Filter paper

Plastic coated spatula

Dessicator

Vacuum pump

1. Turn the fan on in the hood.

2. Lay the aluminized side of the Mylar down on the glass plate making sure that the protective paper is between the glass and the Mylar.

3. Lightly spray up to five of the target rings with the adhesive. Orient the rings so that the large flange faces upward. Spray the adhesive outside the hood.

4. Place the rings on the Mylar, stretching it slightly to make it smooth. Press the ring to insure a secure bond.

5. Use the scalpel to trim around the edge of the rings and transfer the finished backing into petri dishes.

6. Grind the previously spiked and dried sample (see Appendix G) in the agate mortar. (Due to the hardness of the sample, place a piece of filter paper over the top of the sample and press with the pestle from on top. This is to prevent loss of material when small pieces fracture off.)

7. Grind into a very-fine, well-homogenized powder.

8. Place a target backing on the rubber stopper - Mylar side facing upward.

9. With the spatula, distribute approximately 1 mg of the powdered sample into a 6 mm diameter circle in the center of the Mylar.

10. Pipette 10 µl of the 1% Formvar solution onto the surface of the water.

11. After 10 to 20 seconds, pick the Formvar film from the water with the aluminum plate.

12. Turn the film over, so the "dry" side is facing downward.

13. With the scalpel, cut a section out of the film approximately 5 mm from the edge of the hole.

14. Place the film over the target, making sure that the sample is completely covered, but also, that one portion of the aluminized Mylar is not covered by the film.

15. Transfer to a labelled petri dish with the forceps being careful to touch only the edge of the target.

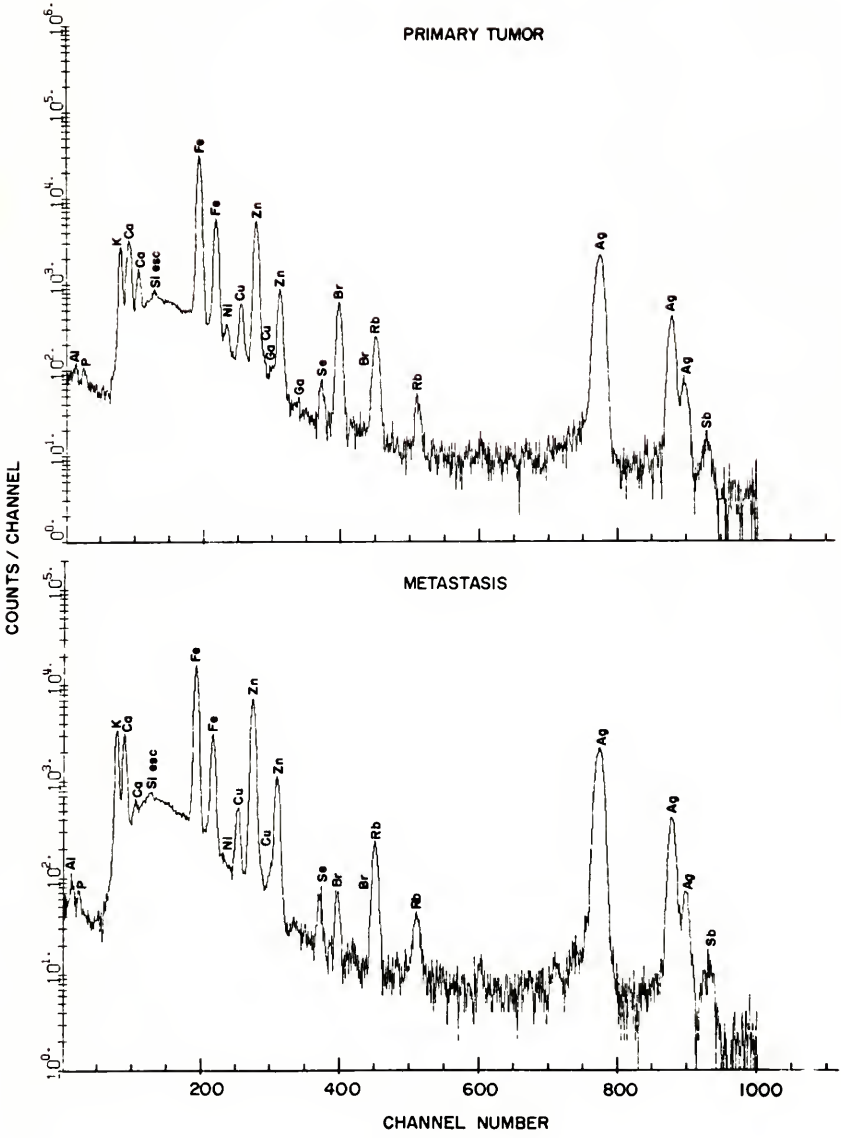
16. After preparing three to five targets, cover the petri dish and place the targets in the dessicator.

17. Connect the vacuum pump to the dessicator and adjust the suction so that the air is evacuated very slowly. This allows air trapped between the film and Mylar to escape without rupturing the film.

18. Thoroughly clean the mortar, pestle, and spatula before grinding and preparing the next sample targets.

APPENDIX I

TYPICAL SPECTRA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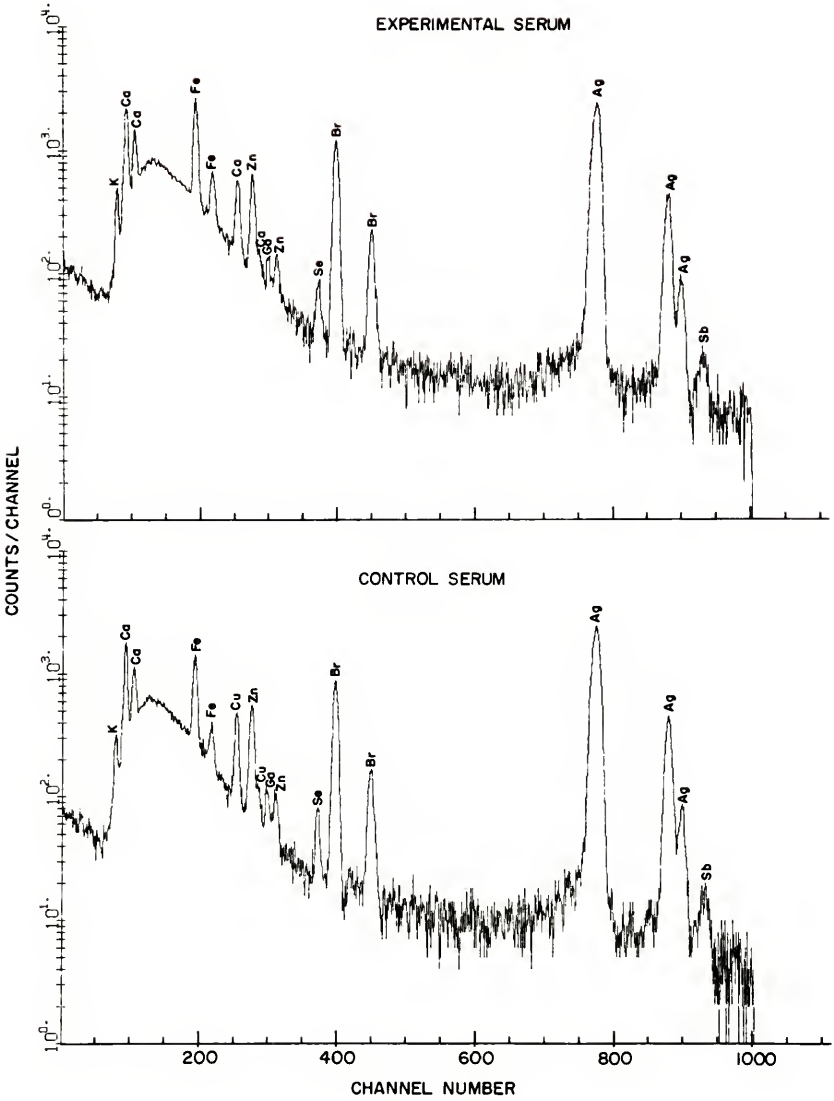
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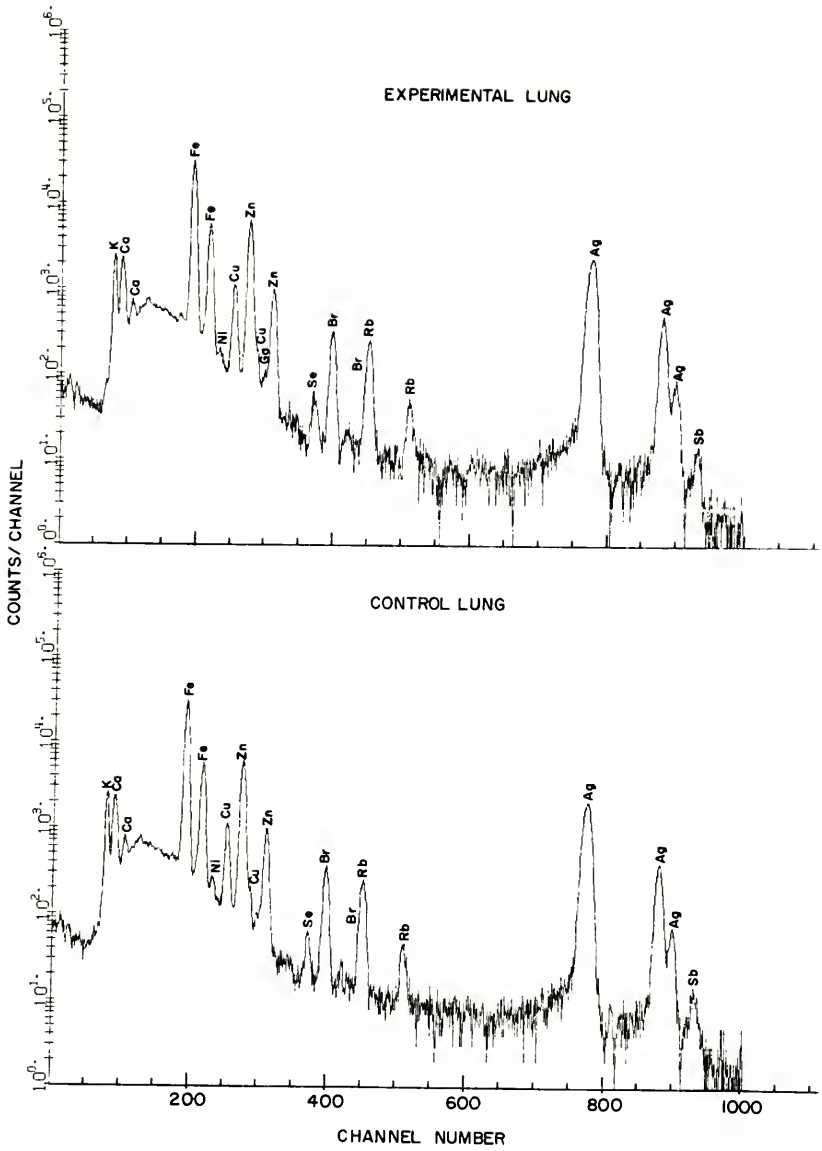
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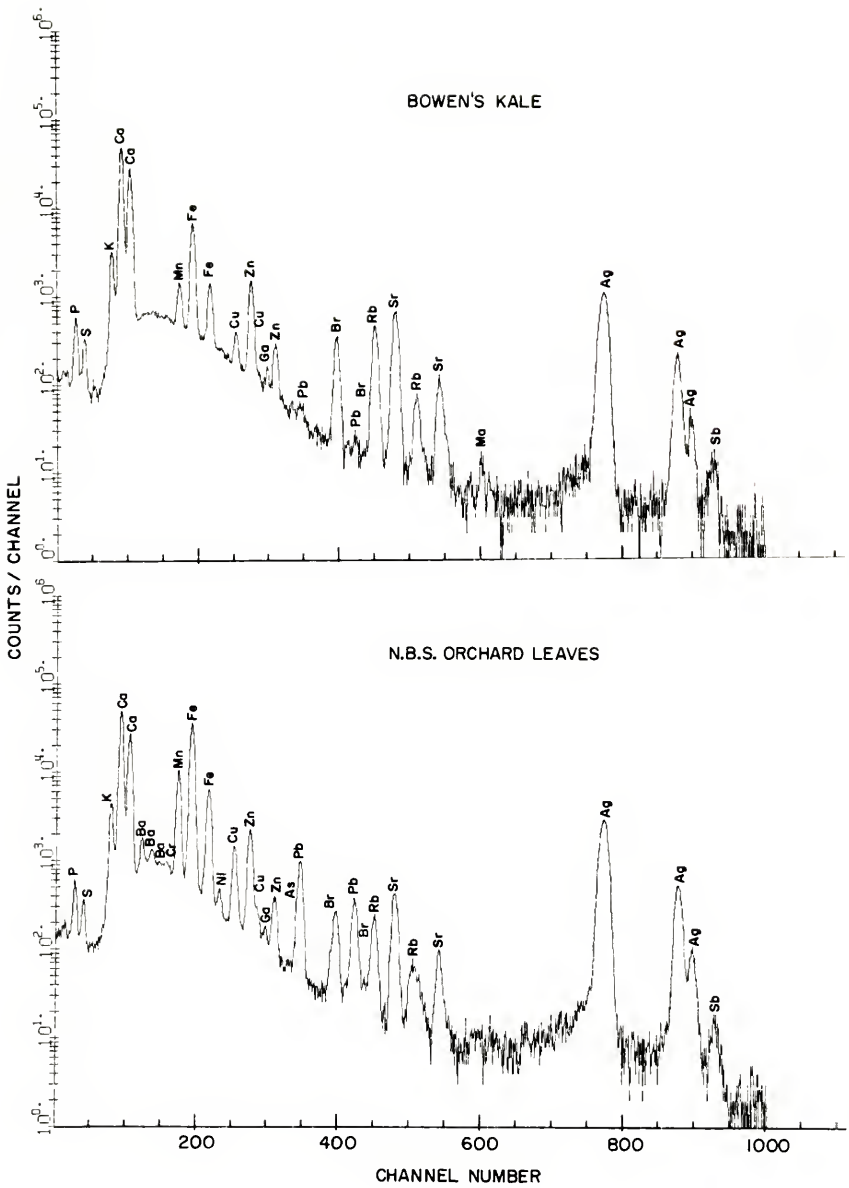
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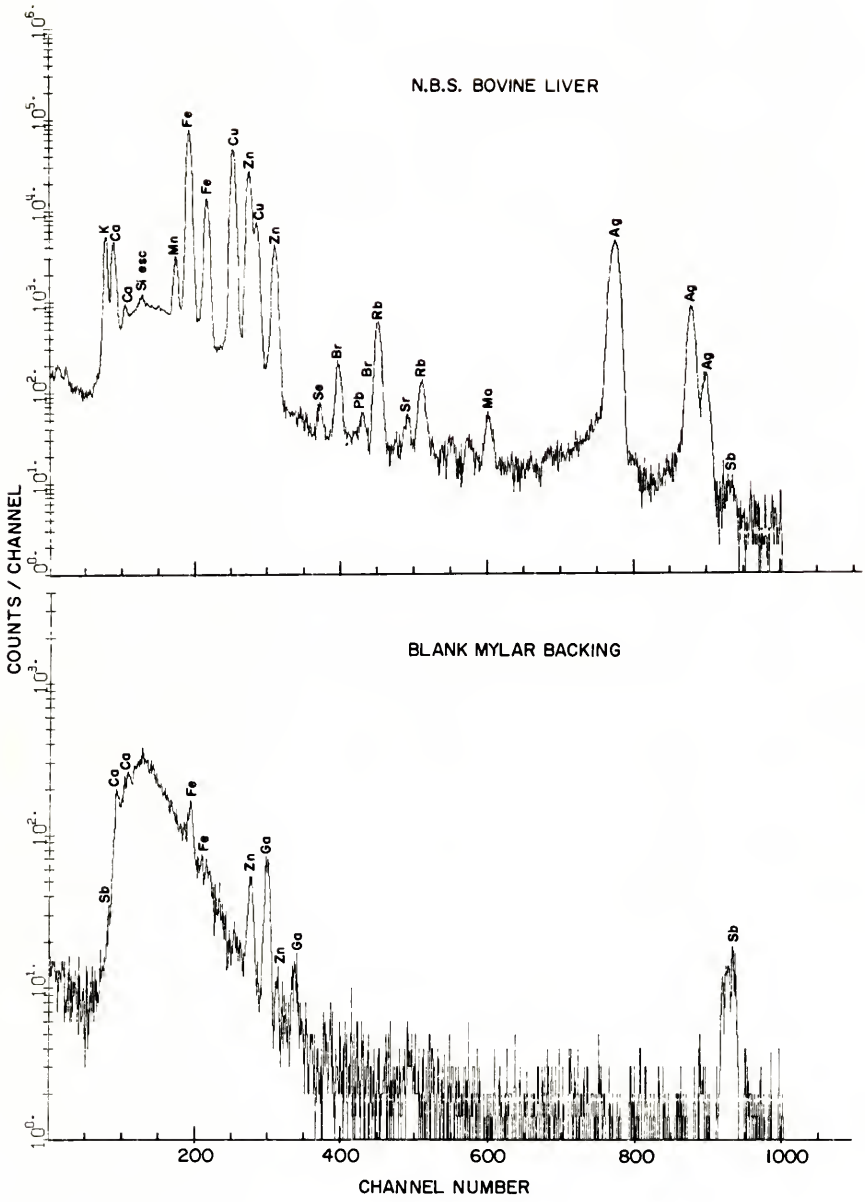
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BIOGRAPHICAL SKETCH

Michael Duncan Williams was born December 27, 1941, in Jacksonville, Florida, where he also received his primary, secondary, and undergraduate education. He graduated from Terry Parker High School in 1959, and received a Bachelor of Arts degree in 1965 from Jacksonville University with a double major in physics and mathematics. In 1966, he was called to serve in the Armed Forces and in March of that year, joined the U. S. Army. He has served on active duty since then. After two years in the Army, he was selected to attend graduate school and in August of 1970, received a Master of Science degree in Nuclear Engineering from the University of Maryland. Following graduation, he was on a special three month assignment with the Defense Atomic Support Agency in Albuquerque, New Mexico, then served thirty-nine months in West Germany with the Nuclear Medical Research Detachment, Europe. His wife, Barbara, and son, Matthew, accompanied him overseas and a second son, Patrick, was born there.

From January through June, 1974, he attended the Officer's Advanced Course at Fort Sam Houston, Texas, then received orders for Tripler Army Medical Center, Honolulu, Hawaii. In this 750 bed hospital, he served as the medical physicist in radiation therapy and as the Radiation Safety Officer for all Army medical units stationed in Hawaii. He remained in this assignment until his selection and transfer to Florida to begin the Ph.D. program in 1977.

He presently holds the rank of Major in the Army and is a member of the American Association of Physicists in Medicine, the Health Physics Society, and the Tau Beta Pi Association.

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

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